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*SOME EFFECTS OF SUBSTITUTING THE DEOXYRIBONUCLEIC ACID OF ISOLATED NUCLEI WITH OTHER POLYELECTROLYTES**

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In previous studies of the synthetic capacities of cell nuclei (isolated in sucrose from calf thymus tissue) it was observed that the removal of the deoxyribonucleic acid from the nucleus is accompanied by a striking loss of biochemical function. Isolated nuclei treated with deoxyribonuclease (DNAase) lose much of their capacity to incorporate amino acids into proteins or purine and pyrimidine precursors into nuclear ribonucleic acids.[1, 2] This loss of activity which accompanies DNAase treatment of the nuclei can be overcome to a considerable extent by adding back a deoxyribonucleic acid supplement. One then observes an apparent restoration of the nuclear capacity to incorporate C^{14}-labeled amino acids into chromosomal proteins, and adenosine-8-C^{14} into nuclear RNAs.

Since the uptake of amino acids is an aerobic phenomenon and depends on the nuclear synthesis of adenosine triphosphate,[3] the results suggested that DNAase treatment might impair the capacity of the nucleus to synthesize ATP. This was found to be the case: nuclei treated with DNAase show a great decrease in their rate of ATP formation, and the addition of a DNA supplement to the treated nuclei permits ATP synthesis to continue.[4] It was concluded that, in a broad sense, the DNA of the intact nucleus could be considered a "cofactor," the presence of which is necessary for the nucleus to carry out aerobic phosphorylation. The failure of DNAase-treated nuclei to incorporate amino acids is in all probability a reflection of the fundamental change in their ATP content.

It was soon found that the restoration of amino acid uptake in DNAase-treated nuclei in the presence of a nucleic acid supplement is a non-specific effect: DNAs from calf tissues other than the thymus and from widely diverse species, DNAs which are denatured and partially degraded, and even ribonucleic acids all showed a general capacity to promote amino acid incorporation into the proteins of DNAase-treated nuclei. The lack of specificity is emphasized by the finding that enzymatically synthesized polyadenylic acid could also replace the nuclear DNA in this isolated system.[4]

On the other hand, it was found that no such effects were obtained when one added RNA hydrolyzed in dilute alkali to yield its component nucleotides. Nor did mixtures of mononucleotides, mononucleosides, or free purine and pyrimidine bases show any effect. ATP and several dinucleotides also failed to promote

amino acid uptake by the DNAase-treated nuclei. Of particular interest was the observation that adenylic-adenylic dinucleotide failed under conditions where polyadenylic acid was effective, suggesting the need for a relatively large polyanionic molecule.

The present paper is concerned with further observations on the loss of function which accompanies DNAase treatment of isolated nuclei. It will be shown that other polyanionic molecules will replace a large part of the DNA in isolated nuclei without impairing amino acid incorporation into protein, adenosine-8-C^{14} incorporation into RNA, or ATP synthesis. It has been found that polyanions such as the polyethylene sulfonates, heparin, and chondroitin sulfate will replace DNA

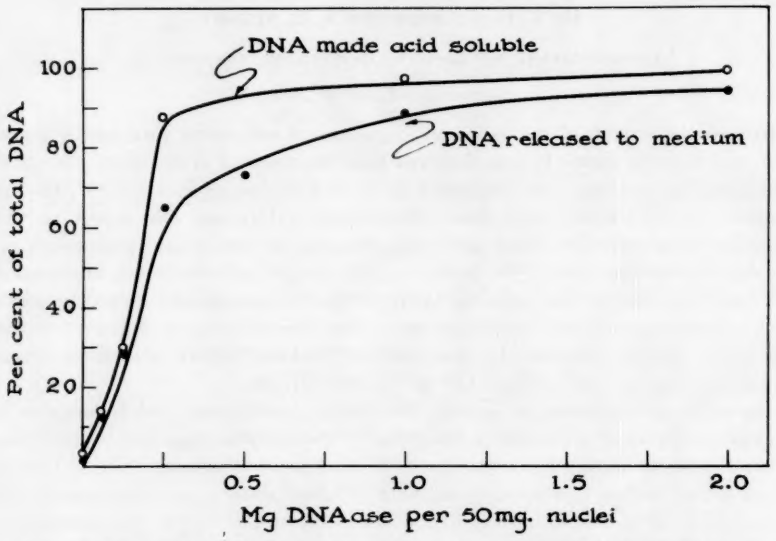

FIG. 1.—The effect of deoxyribonuclease on the DNA content of isolated calf thymus nuclei. All flasks contained 1.0 ml. of nuclear suspension (50 mg. nuclei, dry weight) and 0.4 ml. of 0.1 *M* glucose containing 3.75 mg. NaCl and 4.19 mg. $MgCl_2 \cdot 4H_2O$ per ml. "Control" flasks received 0.5 ml. of 0.1 *M* sodium phosphate and 0.25 *M* sucrose buffer at pH 6.7. Other flasks received this buffer containing the indicated amounts of crystalline pancreatic deoxyribonuclease. (Enzyme concentrations in the buffer ranged from 0.125 to 4.0 mg./ml.) The suspensions were incubated with shaking (100 cycles/minute) at 37° for 30 minutes, and then (1) analyzed for their acid-soluble DNA content (*upper curve*), or (2) the nuclei were centrifuged down and the supernatant analyzed for the DNA released to the medium (*lower curve*).

in this way, and it may be concluded that the effects observed are not limited to nucleotide polymers. Some implications of these experiments which bear on the importance of electrical charge to chromosome structure and DNA function are discussed.

Effect of Deoxyribonuclease on Isolated Thymocyte Nuclei.—When isolated nuclei are suspended in a buffered sucrose medium and exposed to the action of pancreatic deoxyribonuclease, the action of the enzyme can be followed in several ways. First, the release of depolymerized deoxyribonucleic acid into the medium can be determined by centrifuging down the nuclei and analyzing the supernatant fluid. Sec-

ond, the whole suspension can be analyzed for the amount of DNA made acid-soluble by the action of the enzyme. Third, the nuclei may be centrifuged down and their residual DNA content determined by the diphenylamine reaction.

All three methods have been used in determining the extent of DNA degradation and loss which accompany DNAase treatment of isolated thymocyte nuclei. Some of the relationships between enzyme concentration and changes in nuclear DNA are illustrated in Figure 1. In these experiments 50 mg. (dry weight) of nuclei were treated with the indicated amounts of crystalline pancreatic DNAase for 30 minutes at 37° C. (under the conditions described in detail in the legend). It can be seen that high concentrations of the enzyme can depolymerize and release a large part of the nuclear DNA. The "exposed" nature of the isolated nucleus, which makes possible its penetration and attack by DNAase and other enzymes, contrasts with the impermeable nature of intact whole cells, which are resistant to DNAase treatment.[2]

Although crystalline DNAase (Worthington) was used, the actual amount of DNA released or rendered acid-soluble at a given enzyme concentration also depends to a large extent on the particular enzyme preparation used and on the state of the isolated nuclei. In the incorporation experiments to be described below, nuclear suspensions (containing about 50 mg. of nuclei in a volume of 1.9 ml.) were treated with 1 mg. of DNAase for 30 minutes at 37°. On the average, this treatment rendered 74 per cent of the DNA acid-soluble (range 61–93 per cent). If no other polyanions were present during the course of enzyme activity, about 72 per cent of this depolymerized DNA was lost into the medium.

Effect of Polyelectrolytes on DNAase Activity.—The presence of polyelectrolytes in nuclear suspensions exposed to DNAase may modify the activity of the enzyme or change the composition of the nucleus, depending on the charge of the polyelectrolyte.

Polycationic substances such as protamine or polylysine, which carry a net positive charge at neutral pH values, are capable of forming salts with the negatively charged DNA of the nucleus. The presence of these substances during DNAase-treatment leads to a lower yield of depolymerized DNA (Table 1, Expt. A). This effect is due presumably to a shielding of the DNA molecules which prevents their attack by the enzyme.

On the other hand, polyanionic substances such as polyethylene sulfonate, heparin, and chondroitin sulfate, do not appear to inhibit DNAase activity in this system (Table 1, third col.). It can be seen that the amount of DNA made acid-soluble is not diminished by the addition of these negatively charged substances. Moreover, measurements of the release of depolymerized DNA into the medium (Table 1, fourth col.) show that more DNA is lost from nuclei treated with polyanions + DNAase than from nuclei treated with DNAase alone. Thus the polyanion appears to displace some of the depolymerized nucleic acid from sites in the nucleus. The more polyanion added, the greater this displacement (Table 1, Expt. E).

It can be shown that added polyanions actually enter into and remain bound to DNAase-treated nuclei. Some functional tests, which demonstrate such replacement of the nuclear DNA by other molecules, are described below, but more direct chemical tests, which measure the capacity of the DNAase-treated nucleus to bind

added RNA and DNA, have also been carried out. Some of these experiments are summarized in Table 2. In Experiment A, C^{14}-labeled RNA was added to DNAase-treated nuclei, and the amount of label taken up was subsequently measured. It can be seen that when 52.2 mg. of nuclei (containing 13.1 mg. DNA) were treated with 1 mg. DNAase for 30 minutes at 37°, 9.3 mg. (71 per cent) of the DNA was made acid-soluble and 6.3 mg. (48 per cent) was lost into the medium.

TABLE 1
EFFECT OF POLYELECTROLYTES ON DNAASE ACTIVITY
(Tested on Nuclear Suspensions)

Expt.	Compound Added	Amount (Mg.)	DNA Made Acid-soluble (Per Cent of Total)	DNA Lost to Medium (Per Cent of Total)
A	No supplement	..	79.0	54.0
	Polyethylene sulfonate	5	75.0	68.4
	Heparin	5	80.0	69.4
	Protamine	5	66.4	33.0
	Polylysine	5	58.6	30.6
B	No supplement	..	78.4	60.5
	Polyethylene sulfonate	5	81.1	76.2
C	No supplement	..	61.3	43.1
	Polyethylene sulfonate	5	60.9	55.2
D	No supplement	..	72.0	..
	Polyethylene sulfonate	5	71.0	..
	Chondroitin sulfate	5	76.0	..
E	No supplement	..	73.3	50.8
	Polyethylene sulfonate	2	72.0	60.2
	Polyethylene sulfonate	5	74.3	65.2

TABLE 2
BINDING OF ADDED NUCLEIC ACID BY DNAASE-TREATED NUCLEI

Expt.	Compound Added	Amount (Mg.)	Weight of Nuclei (Mg.)	DNA Content of Nuclei (Mg.)	DNA Made Acid-Soluble Mg.	DNA Made Acid-Soluble Per Cent of Total	DNA Lost to Medium Mg.	DNA Lost to Medium Per Cent of Total	Added Nucleic Acid Bound to Nuclei (Mg.)
A	C^{14} Yeast RNA	5.0	52.2	13.1	9.3	71.0	6.3	48.0	1.73
B	Thymus DNA	5.0	44.6	11.2	8.6	77.0	..	..	1.60
	Thymus DNA	10.0	44.6	11.2	8.6	77.0	..	..	2.00
	Denatured Thymus DNA	5.0	44.6	11.2	8.6	77.0	..	..	1.90
	Denatured Thymus DNA	10.0	44.6	11.2	8.6	77.0	..	..	2.40
	Yeast RNA	5.0	44.6	11.2	8.6	77.0	..	..	1.70
	Yeast RNA	10.0	44.6	11.2	8.6	77.0	..	..	3.00

At this point the nuclei were centrifuged down, discarding the supernate, and resuspended in the presence of 5.0 mg. of C^{14}-labeled yeast RNA. After 30 minutes at 37°, the nuclei were centrifuged down, washed with the incubation medium—cold 2 per cent perchloric acid, ethanol, and ether—and their radioactivity was measured. It could be calculated that they had incorporated 34.5 per cent, or 1.73 mg., of the total added C^{14}-RNA. (Since these nuclei had lost 6.3 mg. of DNA, only 27.5 per cent of the loss had been replaced.)

In similar experiments (see Table 2, Expt. B) DNA, denatured DNA, or RNA was added to DNAase-treated nuclei, and the nuclei were subsequently analyzed for their nucleic acid content. It can be seen that all three nucleic acids were taken up and that the quantity bound increases with increasing nucleic acid concentration in the medium.

Effect of Added Polyelectrolytes on Amino Acid and Adenosine Incorporation into Proteins and Ribonucleic Acids of DNAase-Treated Nuclei.—When nuclei are treated with deoxyribonuclease and subsequently given a supplement of DNA, RNA, or other polyelectrolytes, their capacity to incorporate amino acids into proteins, or adenosine-8-C^{14} into nuclear RNAs, will change, depending upon the charge of the polyelectrolyte added.

It has been shown previously that the capacities for incorporation of several different amino acids and of orotic acid can all be restored by a variety of DNAs and RNAs[2] and that polyadenylic acid, too, could "restore" alanine-1-C^{14} and leucine-2-C^{14} uptake in nuclei depleted of their DNA.[4] In more recent experiments, other polyelectrolytes have been tested for their ability to replace the original DNA of the isolated DNAase-treated nucleus. Some of these experiments are summarized in Table 3. The procedure in these tests was as follows: nuclei were incubated in

TABLE 3

EFFECT OF SUPPLEMENTARY POLYELECTROLYTES ON ALANINE-1-C^{14} AND ADENOSINE-8-C^{14} UPTAKE INTO THE PROTEINS AND RIBONUCLEIC ACIDS OF DNAASE-TREATED NUCLEI

		Specific Activity of Nuclear Protein or RNA					
Supplement Tested*	C^{14}-Precursor	"Control" Nuclei (Cpm/Mg)	DNAase-treated Nuclei (Cpm/Mg)	Treated Nuclei + Supplement (Cpm/Mg)	Activity Lost (Cpm/Mg)	Activity Regained (Cpm/Mg)	Recovery of Lost Activity (Per Cent)
1. Calf thymus DNA	Alanine-1-C^{14}	92	54	81	38	27	71
Calf liver RNA	Alanine-1-C^{14}	92	54	80	38	26	68
2. Thymus DNA	Alanine-1-C^{14}	450	167	273	283	106	38
Yeast RNA	Alanine-1-C^{14}	450	167	302	283	135	48
Polyadenylic acid	Alanine-1-C^{14}	450	167	263	283	96	34
3. Thymus DNA	Adenosine-8-C^{14}	4,550	1,510	3,230	3,040	1,720	57
4. Thymus DNA	Alanine-1-C^{14}	229	30	83	199	53	27
Heparin	Alanine-1-C^{14}	229	30	122	199	92	46
Chondroitin sulfate	Alanine-1-C^{14}	229	30	74	199	44	22
Polyethylene sulfonate (MW 27,600)	Alanine-1-C^{14}	229	30	138	199	108	54
Polyethylene sulfonate (MW 12,900)	Alanine-1-C^{14}	229	30	129	199	99	50
Polyethylene sulfonate (MW 5,900)	Alanine-1-C^{14}	229	30	102	199	72	36
5. Thymus DNA	Adenosine-8-C^{14}	2,900	1,260	2,150	1,640	890	54
Polyethylene sulfonate (MW 12,900)	Adenosine-8-C^{14}	2,900	1,260	2,560	1,640	1,300	79
6. No supplement	Alanine-1-C^{14}	259	72	...	187	...	..
Polylysine	Alanine-1-C^{14}	115	72	53	(43)	−19	0
Protamine	Alanine-1-C^{14}	250	72	57	(178)	−15	0
Serum albumin	Alanine-1-C^{14}	267	72	87	(195)	15	7.7

* 5 mg. added in all cases.

the presence of DNAase for 30 minutes at 37°. They were then centrifuged down, discarding the supernate, and resuspended in the incubation medium containing a radioactive precursor (either alanine-1-C^{14} or adenosine-8-C^{14}) plus the added polyelectrolyte. The nuclei were incubated at 37° for 60 minutes, and their protein or RNA was then prepared for counting as previously described.[2, 5] The specific

activity of the protein or RNA in such "supplemented" nuclei was then compared with the corresponding activities of DNAase-treated nuclei which had not received any supplement and also with the activities of "control" nuclei which had not been treated with deoxyribonuclease but which had been incubated, centrifuged, resuspended, and reincubated in the same way (both with and without added polyelectrolyte).

It can be seen that DNAase treatment greatly impairs the capacity of the nucleus for subsequent amino acid or adenosine incorporation. But polyanions, beginning with DNA and including RNA, polyadenylic acid, heparin, chondroitin sulfate, and three molecular sizes of polyethylene sulfonate (PS) (molecular weights 27,600, 12,900, and 5,900) all permit a definite improvement in these nuclear functions. It is evident that there is no specific requirement for a molecule of polynucleotide nature.

The importance of negative charge is emphasized by the results of similar experiments in which polycations, such as polylysine and salmon protamine, were added to DNAase-treated nuclei. Not only did such compounds fail to "restore" amino acid incorporation in DNA-depleted nuclei, but polylysine also seriously inhibited amino acid uptake by "control" nuclei which retained their original DNA content. Added serum albumin, serum globulin, or mixed plasma proteins have no effect on amino acid uptake by DNAase-treated nuclei.

It should be mentioned in passing that citrate ions are added to inhibit further DNAase action during the uptake phase of these experiments. With citrate present, a 55-fold variation in DNAase concentration (0.02–1.10 mg.) in this stage makes no difference to the results obtained. In other tests, diphenylamine analyses for the acid-insoluble DNA content of the nuclei at the end of the experiment showed no significant differences between polyethylene sulfonate–supplemented nuclei and those not receiving added PS.

When restoration experiments are carried out in the above way, first treating with the enzyme and later adding a polyanion supplement, one usually observes that only a part of the lost nuclear activity is recovered. A DNA or RNA supplement will often restore about 70 per cent of the lost activity, but recoveries are sometimes as low as 30 per cent.

DNA Substitution.—On the premise that the removal of the DNA followed by a continued and prolonged incubation would lead to instability and irreversible changes in the state of the chromosomes, attempts were made to stabilize nuclear structure by substituting other polyanions for the DNA at the time of its removal.

It was shown above that the presence of polyethylene sulfonate (PS) during DNAase-treatment does not inhibit the enzyme—on the contrary, it actually displaces some depolymerized DNA from sites in the nucleus. This made it possible to compare the effects of this polyanion when present during DNAase digestion with its later effects when added as a supplement during the uptake phase of the experiment.

Such a comparison is given in Table 4, Experiment A. The procedure in these tests was as follows: nuclei were treated with DNAase in the usual way. In some flasks polyethylene sulfonate was present during this digestion; in others it was not. In either case, the nuclei were centrifuged down and resuspended in incubation medium containing a radioactive precursor (alanine-1-C^{14} or adenosine-8-C^{14}).

TABLE 4

IMMEDIATE AND DELAYED SUBSTITUTION OF NUCLEAR DNA BY POLYELECTROLYTES
(Comparative Effects on Alanine-1-C^{14} and Adenosine-8-C^{14} Incorporation)

	Specific Activity of Nuclear Protein		Specific Activity of Nuclear RNA	
Procedure	Cpm/Mg	Per Cent of Control	Cpm/Mg	Per Cent of Control
A. "Control" nuclei	401	100.0	2,900	100.0
DNAase-treated nuclei*	142	35.7	1,260	43.4
DNAase-treated nuclei, later given 5 mg. DNA	221	55.1	2,150	74.1
DNAase-treated nuclei, later given 5 mg. PS†	308	76.8	2,560	88.3
Nuclei exposed to PS during DNAase treatment	412	102.7	3,220	111.0
B. "Control" nuclei	429	100.0		
DNAase-treated nuclei‡	103	24.0		
DNAase-treated nuclei, later given 5 mg. PS	291	67.8		
Nuclei exposed to PS during DNAase-treatment	432	100.7		
Above nuclei, later given 5 mg. PS	398	92.8		
C. "Control" nuclei	275	100.0		
"Control" nuclei + 5 mg. polylysine	78.4	28.5		
"Control" nuclei + 5 mg. protamine	221	80.4		
DNAase-treated nuclei§	84.8	30.8		
Nuclei exposed to 5 mg. polylysine during DNAase treatment	66.9	24.3		
Nuclei exposed to 5 mg. protamine during DNAase treatment‖	121	...		
Nuclei exposed to 5 mg. heparin during DNAase treatment	271	98.6		
Nuclei exposed to 5 mg. PS during DNAase treatment	286	104.0		

* 61 per cent of DNA made acid-soluble.
† PS = polyethylene sulfonate, MW 12,900.
‡ 59 per cent of DNA made acid-soluble.
§ 79 per cent of DNA made acid-soluble.
‖ Only 66 per cent of DNA made acid-soluble when protamine was present.

For purposes of comparison, nuclei which had not been exposed to polyethylene sulfonate were then divided into two groups, one of which received a PS supplement, and the other did not. A similar division was made for the nuclei which had been treated with PS and for the "control" nuclei (these were not exposed to DNAase action). All the nuclei were then incubated for 60 minutes at 37°, and their protein or RNA prepared for counting.

A comparison of the specific activities of protein and RNA obtained under these different conditions shows that if polyethylene sulfonate is present at the time the DNA is removed, one obtains a nucleus which is the functional equivalent of "control" nuclei whose DNA is still intact. In the experiment cited, 61 per cent of the DNA had been depolymerized by the action of the enzyme, and (with polyethylene sulfonate present) 55 per cent of the DNA was lost into the medium. Yet such nuclei, despite the loss of more than half their DNA, still seem fully normal with respect to their capacity for amino acid and adenosine incorporation. The presence of polyethylene sulfonate at the time the DNA is removed seems to stabilize and protect the synthetic systems involved in such uptake. If under the same conditions one adds the polymer *after* DNAase digestion, only about 70 per cent of the nuclear activity is recovered.

Similar experiments, in which heparin and chondroitin sulfate were present during DNAase-digestion, gave essentially the same results (Table 4, Expt. C). Thus polyanions which by virtue of their charge can substitute for the DNA of the

nucleus at the time of its removal, yield a nucleus which compares very favorably with nuclei whose DNA is still intact. Such effects are not obtained with added polycations, such as protamine or polylysine.

The relationship between the amount of polyethylene sulfonate present during

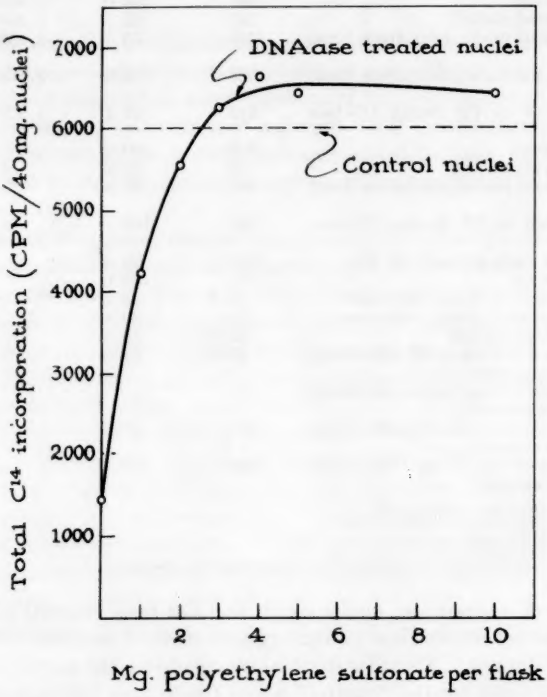

FIG. 2.—The effect of replacing nuclear DNA by increasing amounts of polyethylene sulfonate. All flasks contained 1.0 ml. of nuclear suspension (43 mg. of nuclei) and 0.4 ml. of 0.1 *M* glucose containing 3.75 mg. NaCl and 4.19 mg. $MgCl_2 \cdot 4H_2O$ per ml. plus the indicated amounts of polyethylene sulfonate. "Control" flasks received 0.5 ml. of 0.1 *M* sodium phosphate and 0.25 *M* sucrose buffer at pH 6.7. Other flasks received this buffer containing 1.0 mg. of crystalline DNAase.

After 30 minutes at 37°, the nuclei were centrifuged down and resuspended (using 0.5 ml. of buffer + 0.4 ml. of glucose solution) and added to flasks containing 1.0 ml. of 0.25 *M* sucrose and 0.003 *M* $CaCl_2$ with 2.94 mg. sodium citrate $\cdot 2H_2O$ per ml., and 0.1 ml. of DL-alanine-1-C^{14} solution (0.05 mg. alanine, Sp. Act. 1.4 mc/mM).

After 60 minutes at 37° the nuclear protein was prepared for counting. The total counts incorporated (*ordinate*) are plotted against the amount of polyethylene sulfonate present during the initial DNAase digestion.

DNAase digestion and the subsequent capacity of the nucleus for alanine-1-C^{14} incorporation is illustrated in Figure 2. In these experiments aliquots of a nuclear suspension (43 mg. in 1.9 ml.) were treated with 1.0 mg. of deoxyribonuclease in the presence of the indicated amounts of polyethylene sulfonate (MW 12,900). After

30 minutes at 37° the nuclei were centrifuged down and resuspended in the incubation medium containing alanine-1-C^{14}. The uptake phase lasted 60 minutes, at which time the nuclear protein was prepared for counting. The total counts incorporated into protein are plotted against the amount of PS present during the initial DNAase-digestion. In all cases, regardless of the concentration of PS, 73 per cent of the DNA was made acid-soluble. It can be seen that nuclei given 3–4 mg. of polyethylene sulfonate reached a peak activity actually exceeding that of the "controls," despite the loss and removal of 60–65 per cent of their DNA content.

Effect of Polyethylene Sulfonate on Nuclear ATP Synthesis.—Since amino acid uptake in the nucleus requires the synthesis and utilization of ATP,[3, 4] the above results suggested that polyethylene sulfonate would also restore the ATP-synthesizing capacity of nuclei which had lost this ability as a result of DNAase treatment.[4] Because amino acid incorporation experiments (Table 4) indicated a clear advantage in adding the PS at the time the DNA is removed, rather than later, the experiments were carried out this way. Aliquots of a nuclear suspension were treated with DNAase in the usual manner. In some flasks polyethylene sulfonate (5 mg.) was added to the nuclei; in others it was absent. After 30 minutes at 37° the nuclei were centrifuged down and resuspended in the incubation medium. They were incubated at 37° for 30 minutes. At that time they were centrifuged and extracted with cold 2 per cent $HClO_4$. The nucleotides present in the acid extract were then separated by chromatography on Dowex-1 (formate) by a modification of the technique of Hurlbert *et al.*[4, 6]

A comparison of the ATP peaks obtained from these different nuclear extracts showed that DNAase-treated nuclei had only 20 per cent of the ATP content of the corresponding "controls" (8.7 μg. of ATP per 100 mg. of DNAase-treated nuclei; 43.7 μg. of ATP per 100 mg. of "control" nuclei). In contrast, nuclei given polyethylene sulfonate at the time of DNA removal contained 49.8 μg. of ATP per 100 mg., or 14 per cent more ATP than the controls.

In the experiment cited, 81 per cent of the DNA had been depolymerized by the action of the enzyme, and 76 per cent was lost into the medium.

Thus the nuclear capacity for ATP synthesis, like those for amino acid and adenosine incorporation, can be preserved by adding a polyanion of non-nucleotide nature. This makes it unlikely that the conversion of AMP to ATP which occurs in the isolated nucleus involves the formation of an intermediate complex with the phosphate groups (or the bases) of the deoxypolynucleotide.

In considering mechanisms of nuclear ATP synthesis, the possibility arose that the appearance of ATP in this system might be an indication of polynucleotide breakdown to yield the nucleoside di- or tri-phosphates. This could result from the action of enzymes similar to the polynucleotide phosphorylases described by Grunberg-Manago and Ochoa[7] and by Kornberg and co-workers.[8] Several lines of evidence make this improbable: (1) the action of the polynucleotide phosphorylases is not oxygen-dependent; yet the formation of ATP by the nucleus requires the presence of oxygen. Furthermore, added polynucleotide does not restore ATP synthesis in DNAase-treated nuclei under anaerobic conditions. (2) The enzymatic breakdown of DNA by enzymes similar to those described by Kornberg[8] would yield deoxyribo-ATP rather than the ribose derivative. (3) The ability of polyethylene sulfonate to substitute for the DNA in restoring ATP synthesis argues strongly against a mechanism requiring polynucleotide phosphorolysis.

It should be stressed that in the intact nucleus it is the presence of DNA which makes possible the conversion of AMP to ATP; that DNA is required for the incorporation of amino acids into proteins and for the uptake of adenosine into RNA. Protein and RNA synthesis is, of course, dependent on the nuclear capacity for ATP synthesis, but the fact remains that all these functions in the living nucleus are DNA-dependent. The question which now arises concerns the significance and interpretation of DNA substitution experiments.

DNA Charge and Chromosome Structure and Function.—The preceding experiments have shown that it is possible to remove up to 76 per cent of the DNA of isolated thymus nuclei and to substitute other negatively charged molecules for the missing nucleic acid. This substitution, if properly carried out, yields a completely normal nucleus, as tested by its capacity for ATP synthesis, amino acid incorporation into protein, and adenosine uptake into RNA. The original observations that other polynucleotides would replace DNA in the nucleus have now been extended to include the polyethylene sulfonates, heparin, and chondroitin sulfate.

While these experiments were in progress, there appeared a brief preliminary report by Sekiguchi and Sibatani,[9] describing the effects of DNAase treatment on P^{32} (orthophosphate) incorporation into the deoxyribonucleic acid of isolated rabbit appendix nuclei. In these nuclei, removal of 70–80 per cent of the DNA almost completely abolished the incorporation of P^{32} into DNA and RNA. The addition of a DNA supplement or of chondroitin sulfate both restored activity. The effect of DNAase on P^{32} uptake is thus in accord with earlier observations of the inhibition of thymidine-2-C^{14} uptake into the DNA of rabbit thymus nuclei.[10]

In tests of amino acid or adenosine uptake the substitution of the DNA by other polyelectrolytes is effective only in the case of polyanions. Added polycations, such as polylysine and protamine, do not restore function to nuclei depleted of their DNA. On the contrary, the addition of polylysine to "control" nuclei (which still retain their DNA) greatly inhibits amino acid uptake into nuclear protein. Much of this uptake represents incorporation into the proteins of the chromosome,[2] and the dependence of uptake on charge indicates that electrical charge is one of the main variables involved in chromosome activity.

The experiments described suggest that a pattern of negative charge is correlated with the biochemical activity of the chromosome. Further tests of this hypothesis are now in progress. If it is sustained, it may be possible to demonstrate that one role of the basic proteins (histones and protamines) in the organization of the chromosome is to reduce or control chromosome function by masking the negative charges of the DNA.

One aspect of the DNA substitution experiments which is important to their interpretation concerns the role of the residual DNA which remains in the nuclei even after prolonged incubation at high DNAase concentrations. About 25 per cent of the DNA is not removed under the best conditions employed in the above experiments. (However, much of this residual DNA has been partially depolymerized, and is soluble in acid.) Because this polynucleotide residue remains, one cannot conclude on the basis of substitution experiments that any polyanion is as effective as DNA in stabilizing chromosome structure or mediating chromosome function. Yet it is surprising that, *given an organized nucleus*, more than two-thirds of the DNA can be removed and replaced by a simple polymer such as poly-

ethylene sulfonate, without apparent damage to three fundamental nuclear activities.

Summary.—Up to 75 per cent of the deoxyribonucleic acid of isolated thymocyte nuclei can be removed by incubation with pancreatic deoxyribonuclease. Nuclei so depleted of their DNA lose their capacity for ATP synthesis, for amino acid incorporation into protein, and for adenosine uptake into nuclear RNAs.

The addition of polyanions after DNAase treatment restores much of the biochemical activity of the nucleus. If polyanions are present at the time the DNA is removed, one can substitute for more than two-thirds of the DNA without *any* apparent loss of activity. The polyanions tested include DNAs, RNAs, and polyadenylic acid and non-nucleotides such as polyethylene sulfonate, heparin, and chondroitin sulfate. Added polycations, such as protamine or polylysine, do not restore function to DNAase-treated nuclei. Moreover, polylysine when added to "control" nuclei (whose DNA is still intact) greatly inhibits amino acid uptake into nuclear protein. The findings suggest a correlation between negative charge and the biochemical activity of the nucleus.

We are greatly indebted to the Research Laboratories of the Upjohn Company, Kalamazoo, Michigan, for the gift of the polyethylene sulfonates used in these experiments, and to Dr. Severo Ochoa for supplying enzymatically synthesized polyadenylic acid. It is a pleasure to acknowledge our thanks to Mr. Rudolf Meudt for his careful and expert technical assistance.

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*A NEW WAY FOR THE SYNTHESIS OF 3-AMINO SUGARS**

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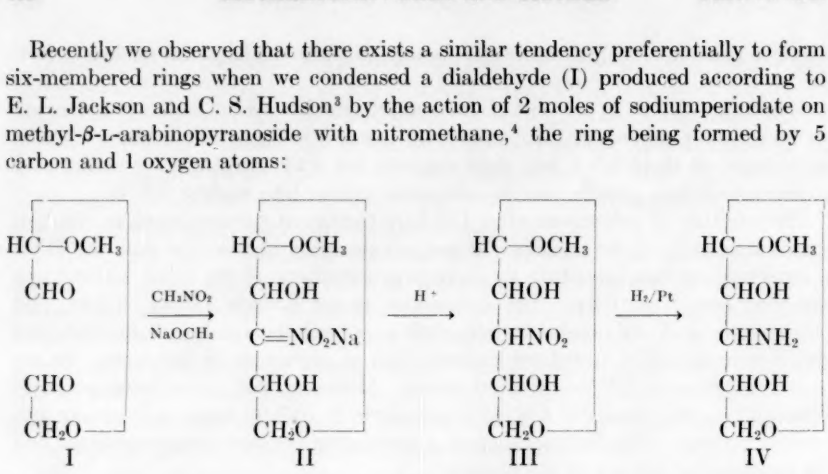
It was found ten years ago that 1,2-acetone-D-xylo-trihydroxyglutaric dialdehyde, a partly blocked dialdehyde of the pentose series, could be condensed with nitromethane and gave, in several steps, in good yield a mixture of nitro-deoxy-inositols.[1] This formation of a six-carbon ring made possible the chemical transformation of D-glucose into myo-inositol.[2]

Recently we observed that there exists a similar tendency preferentially to form six-membered rings when we condensed a dialdehyde (I) produced according to E. L. Jackson and C. S. Hudson[3] by the action of 2 moles of sodiumperiodate on methyl-β-L-arabinopyranoside with nitromethane,[4] the ring being formed by 5 carbon and 1 oxygen atoms:



In other words, in the pyranoside the original CHOH in position 3 was replaced by $CHNO_2$, thus producing 3-nitro-3-deoxy-glycosides, a new class of sugar derivatives. Apparently a twofold condensation of *one* mole of nitromethane with *both* aldehydo groups of the dialdehyde took place rather than condensations of nitromethane with each aldehydo function, even when nitromethane was present in excess.

The reaction yields the expected mixture of 3-nitro-pyranosides out of which one isomer could be isolated as a crystalline sodium salt (II) in a yield of about 40 per cent. Upon catalytic hydrogenation of the free nitro compound (III), we obtained a crystalline methyl-3-amino-3-deoxy-pentoside (IV) (m. p. 189°, $[\alpha]_D = +63.5°$, in water). B. R. Baker and R. E. Schaub[5] found for methyl-3-amino-3-deoxy-β-L-xylopyranoside m. p. 192–193 and $[\alpha]_D = +61.4$, in water. Furthermore, we could prepare a crystalline methyl-3-amino-3-deoxy-pentoside hydrochloride (m. p. 160°, $[\alpha]_D = +119°$ in water) and, by complete hydrolysis, the free amino sugar hydrochloride (crystals, $[\alpha]_D = +18°$ in water).

The analogous series of reactions was applied to a representative of the hexoses, namely, methyl-α-D-glucoside. We obtained in very good yield the condensation product in the form of its sodium salt. The free methyl-3-nitro-3-deoxy-hexopyranoside gave, upon hydrogenation, a corresponding amino derivative which was characterized as its crystalline hydrochloride (decomp. 203°–208°. $[\alpha]_D = +66°$, in water). Configurational assignment was not yet possible because of the lack of compounds of reference.

The experimental details will be published elsewhere.

The wide applicability of the newly discovered pathway of synthesis of 3-amino-sugar derivatives is obvious. Our investigation was prompted by the recently developed interest in 3-amino-sugars, especially in the chemistry of antibiotics. Thus 3-amino-3-deoxy-D-ribose is known to be the sugar moiety of puromycin, and various hexoses and deoxyhexoses carrying a dimethylamino group on carbon atom 3 have been isolated from other antibiotics. Whereas an enormous amount of work has been done on the chemistry of the 2-amino-2-deoxy sugars, relatively little is known of their 3-amino analogues, apart from some older investigations and the recent extensive studies of B. R. Baker and his school.[6]

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THE ENZYMATIC SYNTHESIS OF FATTY ACIDS BY ALDOL CONDENSATION

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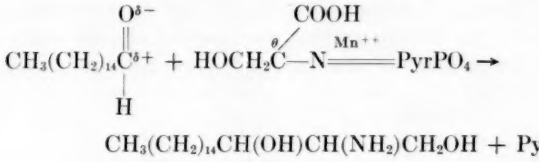
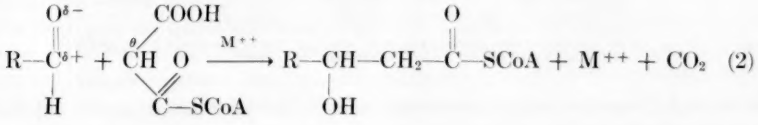
INTRODUCTION

The mechanism of the enzymatic synthesis of sphingosine has recently been established as an aldol condensation of the Knoevenagel type.[1, 2] Such a reaction mechanism had not been previously detected in biological systems. The formation of dihydrosphingosine occurs by a condensation between palmitic aldehyde and the activated methylene group of serine in the presence of pyridoxal phosphate ($PyrPO_4$) and Mn^{++} ions (Reaction 1):



$$CH_3(CH_2)_{14}CH(OH)CH(NH_2)CH_2OH + PyrPO_4 + Mn^{++} + CO_2 \quad (1)$$

The observation that acetaldehyde is a better precursor of fatty acids than acetate[3] and the finding that the addition of malonate caused a marked enhancement of fatty acid synthesis[4] suggested that the formation of fatty acids might also occur by condensation of aliphatic aldehydes with the activated methylene group of malonyl coenzyme A (malonyl CoA) (Reaction 2):



The synthesis of fatty acids has recently been shown to be dependent upon supplemental adenosine triphosphate (ATP), Mn^{++}, and HCO_3^- when acetyl

coenzyme A (acetyl CoA) was used as substrate.[5, 6] Furthermore, it was found that radioactivity from C^{14}-labeled HCO_3^- did not appear in the recovered fatty acids.

These findings are in accord with the hypothesis that acetyl CoA is first carboxylated to form malonyl CoA, which is subsequently decarboxylated during an aldol condensation. The present communication describes recent experiments which indicate that fatty acid synthesis probably occurs via a condensation reaction of the Knoevenagel type.

EXPERIMENTAL

Preparation of Fatty Acid–Synthesizing Enzymes.—The enzyme systems were obtained from pigeon liver as described previously,[7] employing the modifications suggested by Wakil, Porter, and Gibson.[8] The fractions precipitating between 0–30 (Fraction I) and 30–40 (Fraction II) per cent saturation with ammonium sulfate were taken up in 0.04 *M* $KHCO_3$ solution and dialyzed according to the directions of these investigators. For the present studies, the enzyme systems were not further fractionated.

Enzymatic Synthesis of Malonyl CoA.—The experiments of Hayaishi[9] and Beinert and Stansly[10] suggested the presence of a malonic acid–activating system in extracts of *Pseudomonas fluorescens* and acetone-dried preparations of pig-heart tissue. Accordingly, an acetone powder of pig heart was extracted with 10 volumes of a solution of 0.02 *M* $NaHCO_3$, 0.001 *M* versene, 0.001 *M* glutathione, and 0.001 *M* potassium malonate. The fraction precipitating between 40 and 60 per cent saturation with $(NH_4)_2SO_4$ was prepared as described by Green *et al.*[11] This preparation contained a malonate-activating system which was dependent upon the presence of ATP, coenzyme A (CoASH), Mg^{++}, and malonate (Table 1). The product of the reaction was extracted and chromatographed according to the directions of Hayaishi[9] and Stadtman and Barker.[12] A single hydroxamic acid derivative with an Rf of 0.36 was detected when the chromatograms were developed with water-saturated butanol. The relative mobility of this derivative corresponded exactly

TABLE 1*

THE ENZYMATIC ACTIVATION OF MALONIC ACID

Reactant Omitted	Hydroxamic Acid Formed (μmoles)
None	0.32
ATP	0.00
Potassium malonate	0.08
Mg^{++}	0.00
Coenzyme A	0.04

* Except as indicated, the reaction mixtures contained 150 μmoles of tris-(hydroxymethyl)aminomethane hydrochloride buffer (pH 8.2), 10 μmoles of glutathione, 10 μmoles of ATP, 10 μmoles of $MgCl_2$, 75 μmoles of potassium malonate, 0.2 μmole of CoASH, 500 μmoles of hydroxylamine, and pig-heart enzyme (1.5 mg. of protein) in a total volume of 1.2 ml. After incubating at 37° for 30 minutes, hydroxamic acid formation was determined by the method of Lipmann and Tuttle (*J. Biol. Chem.*, *158*, 565, 1945).

with that of monomalonyl hydroxamic acid.[9] Acetohydroxamic acid exhibited an Rf of 0.53 under these conditions.

A large-scale incubation was performed, employing 20 times the quantities of the materials used for the assay system and containing a total of 25 μmoles of CoASH.

The reaction was terminated by the addition of 2 milliequivalents of acetic acid, and the acidified mixtures were heated at 100° for 1 minute. The resulting suspension was centrifuged, and the supernatant solution was taken to dryness by lyophilization. The residue was taken up in a minimal volume of water and applied to a paper chromatogram on Whatman filter paper No. 3. The chromatogram was developed with a solution of an equal volume of ethanol and 0.1 *M* potassium acetate (pH 4.5).[13] After the paper was dried, a small portion was treated with nitroprusside reagent,[13] and a directly staining area was detected with an Rf of 0.35–0.45. Acetyl CoA exhibited an Rf of 0.60 under these conditions. The material was eluted with water from the remainder of the chromatogram, concentrated by lyophilization, and taken up in a small volume of water. A total of 6.4 μmoles of malonyl CoA (purity 30 per cent) was recovered.

Formation of Long-Chain Derivatives of Coenzyme A.—When malonyl CoA was incubated with acetaldehyde. Mn^{++} ions, and the enzymes contained in Fraction I of the pigeon-liver preparations, the synthesis of long-chain acyl derivatives of CoASH could be readily detected (Table 2). The migration of the products of the

TABLE 2*

FORMATION OF LONG-CHAIN DERIVATIVES OF COENZYME A

REACTANT OMITTED	LONG-CHAIN HYDROXAMIC ACIDS FORMED Fraction I (μmoles)	Fraction II (μmoles)
None	0.102	0.097
Acetaldehyde	0.00	0.00
Malonyl CoA	0.00	0.00
Mn^{++}	0.00	0.00
Malonyl CoA replaced with 1.0 μmole of acetyl CoA	0.00	0.00

* Except as indicated, the reaction mixtures contained 40 μmoles of L-histidine hydrochloride buffer (pH 6.5) (Wakil *et al.*, *Biochim. et Biophys. Acta*, **24**, 453, 1957), 50 μmoles of acetaldehyde, 0.5 μmole of $MnCl_2$, 1.0 μmole of malonyl CoA, 0.1 μmole of triphosphopyridine nucleotide, and enzymes contained in Fraction I or II of the pigeon-liver extracts (10 mg. of protein) in a total volume of 0.78 ml. The tubes were stoppered and incubated at 37° for 30 minutes. The reaction was terminated by the addition of 500 μmoles of hydroxylamine hydrochloride. After standing for 10 minutes at room temperature, 6 volumes of ethanol were added, the mixtures centrifuged, and the supernatant solutions evaporated to dryness. The residue was extracted with 2 ml. of acetone, concentrated to 0.1 ml. under nitrogen, and applied to paper chromatograms, using water-saturated butanol or mixtures A or B of Benson *et al.* (*J. Am. Chem. Soc.*, **72**, 1710, 1950). After the solvent front had advanced 35 cm., portions of the chromatograms were sprayed with 2.5 per cent $FeCl_3$ in ethanol. The products of the reaction with Fraction I with an Rf value of 0.87 were eluted from the butanol-H_2O chromatography system, and the amount of hydroxamic acid was determined quantitatively. When the enzyme system contained in Fraction II was employed, the products with an Rf of 0.94 were eluted, and the hydroxamic acid content was determined. In this chromatography system, the Rf values for the hydroxamic acid derivatives of acetic acid, β-hydroxy butyric acid, crotonic acid, and butyric acid were 0.51, 0.73, 0.75, and 0.80, respectively.

reaction in three separate solvent systems[12, 14] corresponded with hydroxamic acid derivatives with chain lengths between 8 and 12 carbon atoms. When malonyl CoA was incubated under these conditions with the enzymes contained in Fraction II, the hydroxamic acid derivatives of the products of the reaction migrated to regions corresponding to that of fatty acids with chain lengths between 14 and 18 carbon atoms. A clear dependence upon malonyl CoA, acetaldehyde, and Mn^{++} was found. Although the results shown in the tables are taken from individual experiments, almost identical findings were obtained in at least two confirmatory experiments with each enzyme preparation. Acetyl CoA could not replace malonyl CoA for the formation of long-chain derivatives, although both fractions catalyzed the formation of β-hydroxy butyryl CoA from acetyl CoA, probably because of the presence of the enzyme β-keto thiolase.[15]

Enzymatic Reduction of Acetyl CoA.—If the initial reaction for fatty acid synthesis be a condensation between acetaldehyde and malonyl CoA, it would follow that acetyl CoA must be reduced to the level of the aldehyde prior to the condensation. The reduction of acetyl CoA is catalyzed by an enzyme present in extracts of *C. kluyveri* which utilizes reduced diphosphopyridine nucleotide (DPNH),[16] and the reduction of palmityl CoA is catalyzed by an enzyme system obtained from rat-brain tissue which requires reduced triphosphopyridine nucleotide (TPNH).[1] It was demonstrated in the present study that Fraction I obtained from pigeon-liver tissue contains an enzyme which catalyzes the reduction of acetyl CoA in the

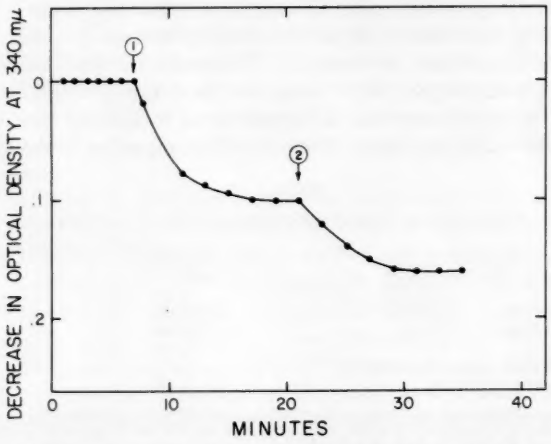


FIG. 1.—Spectrophotometric measurement of the reaction between acetyl CoA and TPNH. The reaction mixtures contained 100 μmoles of potassium phosphate buffer (pH 7.0), 10 μmoles of semicarbazide, 1.3 μmoles of KCN, 0.5 μmole of $MnCl_2$, 0.13 μmole of TPNH, and the enzymes contained in Fraction I of the pigeon-liver preparation (6 mg. of protein) in a total volume of 1.0 ml., d = 1.0 cm. At arrows *1* and *2*, 0.2 μmole of acetyl CoA was added. The temperature was 22°. The readings were corrected for dilution after the additions and for the slight endogenous oxidation of TPNH which occurred in the absence of acetyl CoA.

presence of TPNH (Fig. 1). In additional experiments without semicarbazide, this reaction was found to be reversible, as has been observed with the comparable reactions in the other enzyme preparations. Furthermore, the reduction of acetyl CoA occurred at a much greater rate in this system in the presence of malonyl CoA (Fig. 2).

DISCUSSION

The results of the present experiments are consistent with a mechanism in which the formation of long-chain fatty acids occurs by the successive condensation of aliphatic aldehydes with malonyl CoA in the presence of Mn^{++} ions. The aliphatic aldehydes are produced by the reduction of the respective thiol ester derivatives of coenzyme A in the presence of TPNH. The aldehydes condense with the activated

methylene carbon atom of malonyl CoA, and the free carboxyl carbon atom of malonyl CoA is displaced in the course of the reaction. The product of the condensation reaction would be a β-hydroxy derivative of coenzyme A which could be dehydrated and then reduced with a second molecule of TPNH.[17] Acetaldehyde may perform a dual role in the system employed in the present investigations by initiating the condensing reaction with malonyl CoA as well as being a substrate for the generation of TPNH for the saturation of ethylenic derivatives and reduction of thiol esters. The necessity for ATP for the conversion of acetyl CoA to fatty acids[5, 6] may be due to the requirement of this nucleotide for the activation of carbon dioxide.[18] Malonyl CoA may be formed from acetyl CoA, ATP, and CO_2 in a reaction resembling the carboxylation of propionyl CoA to form methylmalonyl coenzyme A.[19]

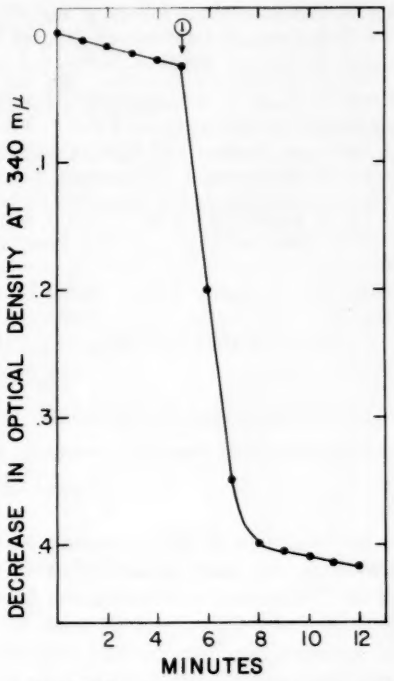


FIG. 2.—Spectrophotometric measurement of the effect of malonyl CoA on the reaction between acetyl CoA and TPNH. The reaction mixtures contained 150 μmoles of potassium phosphate buffer (pH 7.0), 0.2 μmole of TPNH, 0.5 μmole of $MnCl_2$, and the enzymes contained in Fraction I of the pigeon-liver preparation (6 mg. of protein) in a total volume of 1.5 ml., d = 0.5 cm. The reaction was initiated by the addition of 0.26 μmole of acetyl CoA. At arrow *1*, 0.2 μmole of malonyl CoA was added. The temperature was 22°. The readings were corrected for dilution after the additions.

SUMMARY

The synthesis of fatty acids was studied in enzyme systems obtained from pigeon-liver tissue.

The formation of long-chain derivatives of coenzyme A occurs from acetaldehyde and malonyl coenzyme A in the presence of reduced triphosphopyridine nucleotide and Mn^{++} ions. Adenosine triphosphate is not required.

A mechanism for the synthesis of fatty acids is proposed on the basis of these findings.

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*PHOSPHORYLATED INTERMEDIATES IN THE SYNTHESIS OF SQUALENE**

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Communicated July 31, 1958

Investigations in this laboratory on the mechanism of squalene synthesis from mevalonic acid have proceeded along two lines: (1) certain structural features of the "isoprenoid" condensing unit have been defined by experiments using heavy hydrogen as a tracer, and (2) yeast extracts have been fractionated with the aim of accumulating intermediates between MVA[1] and squalene. The experiments with D_2O[2] and T-labeled MVA[3] have led to the principal conclusions that the new carbon-carbon bonds between two isoprenoid units are formed by the interaction of two methylene groups and that the carboxyl groups of MVA are probably removed in a concerted process with simultaneous elimination of the tertiary hydroxyl groups. As to the requirements for the over-all process, it could be shown that in yeast extracts the co-factors for squalene synthesis from MVA are ATP, Mn^{++}, and reduced pyridine nucleotide. To account for the requirements of ATP and manganese, the formation of phosphorylated intermediates was postulated.[3] The subsequent demonstration of an enzyme system that catalyzes the reaction MVA + ATP → phospho-MVA + ADP confirmed this hypothesis.[4] Phospho-MVA (P-MVA) is efficiently converted to squalene and appears to be the first product in the over-all transformation. Evidence has been presented that P-MVA is a phosphate ester[4] and, as will be shown later, can be assumed to be 5-phosphomevalonic acid. We have also reported briefly[6] that ATP is still required for the further metabolism of P-MVA, suggesting the existence of additional phosphorylation steps. We now wish to describe a second and a third phosphorylated intermediate (II and III) which are formed during the conversion of MVA to squalene.

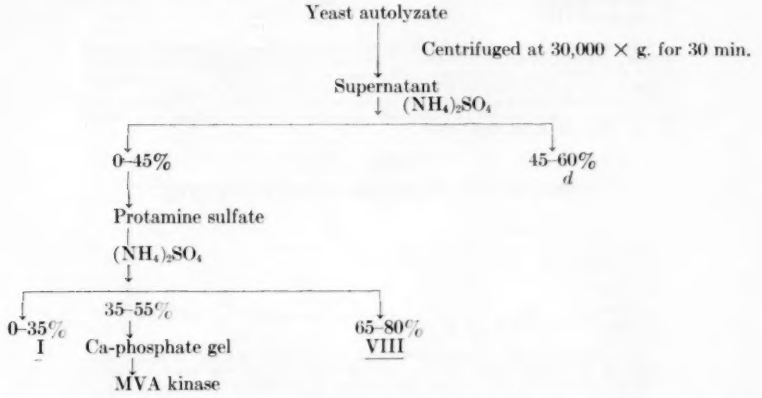
EXPERIMENTAL

Materials.—Samples of 1-C^{14}-MVA and 2-C^{14}-MVA were generously furnished by the Merck, Sharpe & Dohme Laboratories. One sample of 2-C^{14}-MVA was purchased from the Isotope Specialties Co., Burbank, California. The 1-C^{14}-

MVA used in later experiments was synthesized by H. Rilling according to Cornforth *et al.*[7] ATP^{32} was prepared by incubation of rat liver homogenates with AMP and radioactive inorganic phosphate[8] and purified by chromatography.[9] C^{14}-ATP, labeled in the adenine moiety, was kindly provided by Dr. Paul Zamecnik. Samples of P^{32}-MVA, 1-C^{14}-P-MVA, and 2-C^{14}-P-MVA were prepared by incubation of the appropriately labeled MVA with ATP (or ATP^{32}) and Mn^{++} in the presence of MVA kinase.[5] This enzyme was prepared as described later.

Methods.—The solvent system used for the chromatographic separation of MVA derivatives was *t*-butanol–formic acid–water (40:10:16). The R_f values observed in this system were: P-MVA, 0.55; compound II, 0.34; and compound III, 0.65.

Preparation of Enzymes.—Autolyzates of dried baker's yeast (Fleischmann), prepared as previously described,[3] were fractionated, as shown below, to yield MVA-kinase and the enzyme fractions designated I, VIII, and *d:*



Incubation Experiments.—The appropriate enzyme fractions were incubated with ATP (0.005 *M*), Mn^{++} (0.001 *M*), and P-MVA at 30° for the desired lengths of time. Reactions were terminated by immersing the reaction flasks in boiling water for 1 minute. Precipitated protein was removed by centrifugation, and the supernatant fluid applied to strips of Whatman paper No. 1 or 3 for chromatography. The radioactive areas were located by counting in an Actigraph (Nuclear, Chicago). When necessary, the radioactive areas were eluted with water and rechromatographed. Samples containing both C^{14} and P^{32} were counted in a gas-flow counter both without and with an aluminum window, which reduced the counting efficiency for C^{14} to 0.05 per cent and for P^{32} to 50 per cent.

The decarboxylation of 1-C^{14}-MVA derivatives was followed by trapping evolved CO_2 and counting it as $BaCO_3$. For stability studies, samples of compounds II and III were heated in 1 *N* HCl or 1 *N* KOH for 10 minutes in a boiling water bath. Samples that had been treated with alkali were acidified by addition of Dowex-50-H^+. All samples were evaporated to dryness *in vacuo* and chromatographed.

RESULTS AND DISCUSSION

Following the isolation of P-MVA, it was demonstrated that this phosphate ester is converted to squalene[4] but that ATP and Mn^{++} or Mg^{++} are still necessary

for this conversion (Table 1). The same ATP dependence is observed for the decarboxylation reaction as measured by the release of $C^{14}O_2$ from 1-C^{14}-P-MVA (Table 2). This clearly indicated that further phosphorylations occur during the transformation of P-MVA to squalene. To demonstrate the suspected phosphate esters, P-MVA was incubated with enzyme VIII, ATP, and Mn^{++} for various time periods, and the products separated by chromatography. The results are shown in Figure 1. As P-MVA is being consumed, two new peaks appear, one

TABLE 1*

EFFECT OF ATP ON SQUALENE SYNTHESIS FROM MVA DERIVATIVES

SUBSTRATE	SQUALENE (c.p.m.) With ATP	SQUALENE (c.p.m.) Without ATP
P-MVA	206	4
Compound II	235	4
Compound III	51	241

* The assay system contained 0.01 ml. of enzyme fraction I (3.3 mg. of protein); 0.05 ml. of enzyme fraction *d* (4.4 mg. of protein); 0.1 ml. of KH_2PO_4 buffer, 0.2 *M*, pH 7.4; 1,000 c.p.m. of substrate; 0.2 μmoles of Mn^{++}; ATP when present, 2.3 μmoles; in a total volume of 0.7 ml. After incubation for 1 hour at 30°, 1 mg. of DPNH was added and incubation continued for another hour. Squalene was isolated and characterized as previously described (Amdur, Rilling, and Bloch, *J. Am. Chem. Soc.*, **79,** 2646, 1957).

TABLE 2*

EFFECT OF ATP ON DECARBOXYLATION OF P-MVA

	CO_2 (c.p.m.)
Complete	158
− ATP	4
− Mn^{++}	12
− Mn^{++} + Mg^{++}	163
− Mn^{++} + EDTA (5×10^{-3} *M*)	0

* The assay system contained 0.05 ml. of enzyme *d* (4.4 mg. of protein); 0.01 *M* phosphate buffer, pH 7.6; 0.01 *M* KF; 5×10^{-4} *M* Mn^{++}; ATP when present, 1×10^{-3} *M*; and 20 μmoles of 1-C^{14}-P-MVA (500 c.p.m.). Incubation was carried out for 30 minutes at 30°.

less mobile (II) and the other more mobile (III) than P-MVA. After short periods of incubation, only II accumulates, indicating that it is the precursor of III. After longer incubation, two additional, even faster-moving peaks (IV and V) are observed, but these have not been further investigated. In order to characterize compounds II and III, a series of experiments were carried out, using 1-C^{14}-P-MVA, 2-C^{14}-P-MVA, 2-C^{14}-P^{32}-MVA, and P^{32}-MVA as substrates, and unlabeled ATP as well as C^{14}- and P^{32}-labeled ATP as co-factors. By combining various labeled substrates and labeled or unlabeled ATP, the following properties of compounds II and III were established:

1. When either P^{32}-MVA or 2-C^{14}-P-MVA are the substrates, both compounds II and III appear on the chromatograms.

2. When 1-C^{14}-P-MVA is used, only II, but not III, is observed, regardless of the length of incubation. Compound III therefore lacks the carboxyl group of MVA, which is in line with the observation that fraction VIII also catalyzes the decarboxylation of 1-C^{14}-P-MVA.

3. After incubation of P^{32}-MVA and C^{14}-labeled ATP, compounds II and III contain P^{32} but not C^{14}, showing that they are not adenine nucleotide derivatives.

4. When 2-C^{14}-P^{32}-MVA is incubated with unlabeled ATP, both II and III

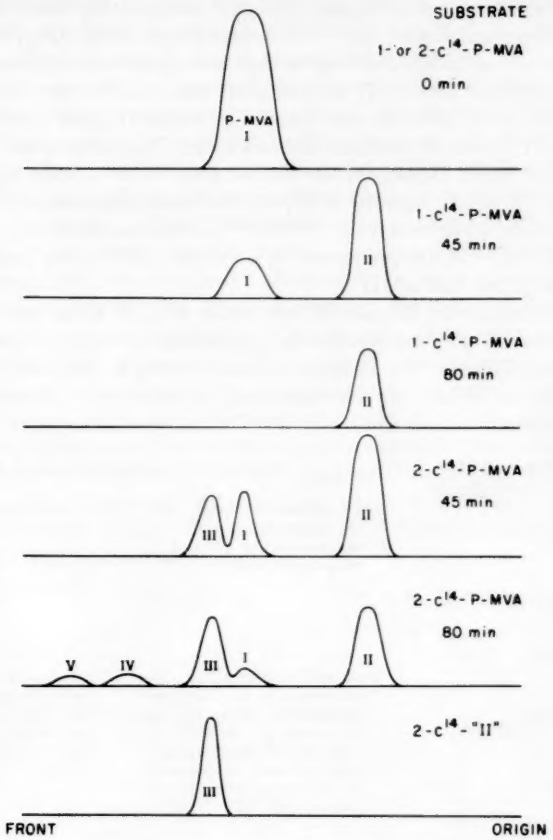


FIG. 1

TABLE 3

C^{14}/P^{32} RATIOS IN PRODUCTS FORMED FROM P-MVA AND ATP

Reaction	Compound Isolated	C^{14}/P^{32}
1. 2-C^{14}-P^{32}-MVA + ATP	II	0.88
	III	0.95
2. 2-C^{14}-P-MVA + ATP^{32}	II	(*a*) 0.74
		(*b*) 0.89
	III	(*a*) 1.06
		(*b*) 0.75
3. P^{32} − MVA + ATP-C^{14}	II	No C^{14}
	III	No C^{14}
4. 2-C^{14}-II + ATP^{32}	III	No P^{32}

are found to contain P^{32} and C^{14} in the same ratio as the starting material (expt. 1, Table 3). The phosphate group of P-MVA is therefore retained in both products.

5. When 2-C^{14}-P-MVA was incubated with ATP^{32}, compounds II and III contained P^{32} as well as C^{14}; in both compounds the molar ratio of the two isotopes was again close to unity (expt. 2, Table 3). Therefore, in the conversion of P-

MVA to II and III, a new phosphate group is incorporated from ATP. Since it has already been established that both intermediates retain the phosphate of P-MVA, it must be concluded that they contain two phosphate residues per molecule.

6. When carboxyl-labeled II is incubated with the enzyme, radioactive CO_2 is produced. Under the same conditions C_2-labeled II gives rise to radioactive III, and hence III must be the later intermediate. This sequence is also indicated by the order in which II and III are formed from P-MVA (Fig. 1). The decarboxylation of II to III requires ATP, which cannot be replaced by ADP. In this step, however, label from ATP (C^{14} or P^{32}) is not introduced into the product.

7. Both II and III are precursors of squalene, ATP being required for the conversion of II but not of III.

Stability.—Compounds II and III are stable to 1 *N* KOH for 10 minutes at 100° C. However, they are completely hydrolyzed by heating with 1 *N* HCl at 100° C. for 10 minutes. The products of acid hydrolysis obtained from variously labeled samples of II and III were analyzed by paper chromatography with the following results:

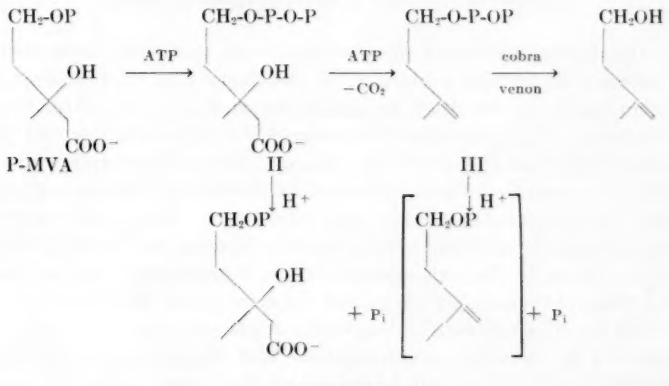
Substrates for the Preparation of II and III	Products of Acid Hydrolysis from II	Products of Acid Hydrolysis from III
CH_2-OP* | CH_2 — OH | C / \ * and ATP CH_3 CH_2 | COOH	One radioactive peak with R_f identical to P-MVA and containing P^{32} and C^{14} in a molar ratio of 1:1	One radioactive peak with a slightly higher R_f than inorganic phosphate; no labeled inorganic phosphate is formed
CH_2-OP | CH_2 — OH and AMP-P*-P* C / \ * CH_3 CH_2 | COOH	Two radioactive peaks; one corresponds to inorganic phosphate; the other has the same R_f as P-MVA; this second peak contains only C^{14} and is converted to squalene only in the presence of ATP	Two radioactive peaks, one being inorganic phosphate, the other contains only C^{14} and has an R_f value slightly higher than inorganic phosphate

Thus the principal chemical change that is observed on treatment of II or III with acid is the removal of one of the two phosphate residues. The labile phosphate is the one which is introduced in the reaction between P-MVA and ATP. By contrast, the phosphate carried over into II and III from P-MVA is resistant to acid hydrolysis.

The incorporation of C^{14} and P^{32} and the acid lability of II and III are interpreted as follows: P-MVA reacts with ATP to form a mevalonic acid derivative (II) with the introduction of another phosphate group. The newly introduced phosphate residue is acid-labile, and, when it is split off, a compound having the same properties as P-MVA is formed. Thus the structure of II differs from that of P-MVA only by the presence of an additional phosphate group. Compound II is converted to III with the elimination of CO_2, and in this process both phosphate residues are retained. The acid stability of the two phosphate residues in III is the same as in II. Although decarboxylation of II to III requires ATP, none of the elements of the nucleotide are introduced into III during this reaction.[10]

As pointed out in the introduction, we have postulated[2] that the decarboxylation

reaction is accompanied by the removal of the tertiary hydroxyl group at C_3 of MVA, with the formation of a double bond between C_2 and C_3. It follows from this argument that III, which is a decarboxylation product, no longer contains this tertiary alcohol group. Thus only the primary alcohol function at C_5 remains for attachment of the two phosphate groups, and, barring the unlikely alternative that it is a C_{10} compound with four phosphate residues, III must be the pyrophosphate ester of an unsaturated 5-carbon alcohol. This conclusion is supported by the properties of a product that is formed on removal of the phosphate residues by enzymatic hydrolysis. When C^{14}-labeled III was incubated with cobra venom as a source of phosphatases and the reaction mixture steam-distilled, a volatile radioactive compound was obtained. After addition of 3-methyl-but-3-ene-1-ol (Δ^3-isopentenol) as carrier, the 3,5-dinitrobenzoyl derivative was prepared and recrystallized. Radioactivity failed to separate from the carrier, indicating that the volatile hydrolysis product of III is indeed Δ^3-isopentenol. While more direct chemical evidence is necessary for final structural proof, it is clear at any rate that compound III is the pyrophosphate ester of an unsaturated 5-carbon alcohol. A consideration of the chemical and enzymatic relationships between the three phosphate derivatives, moreover, suggests that compound II is mevalonic acid-5-pyrophosphate and that the phosphate group of P-MVA is esterified with the primary hydroxyl at C_5. The enzymatic and chemical properties of these compounds are summarized as follows:



SUMMARY

Two new phosphate derivatives of mevalonic acid, both containing two phosphate residues per molecule, are described. The structures tentatively assigned to these intermediates in squalene synthesis are mevalonic acid-5-pyrophosphate and Δ^3-isopentenol pyrophosphate, respectively.

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† Damon Runyon Cancer Research Fellow.

‡ Holder of a Postdoctoral Fellowship of the National Science Foundation.

§ Holder of a Postdoctoral Fellowship of the United States Public Health Service.

|| American Cancer Society Scholar.

[1] The following abbreviations are used: MVA, mevalonic acid; P-MVA, phosphomevalonic acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

[2] H. Rilling, T. T. Tchen, and K. Bloch, these PROCEEDINGS, **44**, 167, 1958.

[3] B. H. Amdur, H. Rilling, and K. Bloch, *J. Am. Chem. Soc.*, **79**, 2646, 1957.

[4] T. T. Tchen, *J. Am. Chem. Soc.*, **79**, 6344, 1957.

[5] T. T. Tchen, *J. Biol. Chem.* (in press).

[6] A. H. Phillips, T. T. Tchen, and K. Bloch, *Fed. Proc.*, **17**, 289, 1958.

[7] J. W. Cornforth, R. H. Cornforth, G. Popjak, and I. Youhotsky-Gore, *Biochem. J.*, **69**, 146, 1958.

[8] S. P. Colowick and N. O. Kaplan, *Methods of Enzymology*, **4**, 853, 1957.

[9] R. B. Hurlbert, H. Schmitz, A. F. Brumm, and V. R. Potter, *J. Biol. Chem.*, **209**, 23, 1954.

[10] A possible explanation of the role of ATP in the transformation of II to III is that it gives rise to additional phosphorylated products, which, however, are too rapidly converted to III to be detected.

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*THE MODE OF ACTION OF 5-FLUOROURACIL AND ITS DERIVATIVES**

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In 1954 this laboratory described experiments on the lethal consequences of thymine deficiency in growing bacteria.[1] It was shown that when various strains of *Escherichia coli* were permitted to metabolize and grow under conditions of thymine deficiency, which prevented the synthesis of deoxyribonucleic acid (DNA), the cells irreversibly lost the power to multiply. In subsequent explorations of this phenomenon it was found that additional nutritional deficiencies, e.g., purine,[2] amino acids,[3] uracil,[4] tended to minimize this lethal effect. In our early experiments it proved to be relatively difficult to induce specific thymine deficiencies by means of competitive analogues.[2] Thus an analogue such as 5-bromouracil was incorporated into DNA in place of thymine and permitted the synthesis of this polymer in modified form when thymine was entirely omitted from the medium.

It was pointed out in earlier communications that the phenomenon of thymineless death which resulted from unbalanced growth bore some similarities to certain aspects and types of cellular differentiation[5] and that the thymineless bacteria possessed numerous cytological and chemical similarities to bacteria killed by a variety of bactericidal agents or to tumor cells killed by a number of antitumor agents, e.g., amethopterin.[6] It was suggested that the specific induction of thymine deficiency to kill cells might be of chemotherapeutic interest in a number of biological systems, particularly that of tumors.[2]

In the search for antitumor agents, Heidelberger, Pleven, and Duschinsky[7] have prepared 5-fluorouracil, hoping to take advantage of the high rate of uracil utilization in tumors[8] in affecting nucleic acid biosynthesis with this analogue. It has been observed[9, 10, 10a] that 5-fluorouracil, and particularly its deoxyriboside, possessed unusual antitumor activity and that these compounds affected the biosynthesis of

thymine in bacterial and tumor systems. In addition, 5-fluorouracil is incorporated into RNA to a significant extent as a uracil analogue.

In the spring of 1957 these workers invited this laboratory to participate in the study of the effects of these compounds in bacterial systems. In this paper we shall present data on the effects of these compounds on growing and virus-infected bacteria. We have shown that the most potent of the fluorouracil derivatives, 5-fluorouracil deoxyriboside, induces thymineless death in *E. coli*. The deoxyriboside is converted in the bacteria to 5-fluorouracil deoxyribotide. This nucleotide has been isolated from the acid-soluble fraction of *E. coli* exposed to the nucleoside; it has also been synthesized enzymatically. This nucleotide has been found irreversibly to inactivate thymidylate synthetase, an enzyme which we have isolated from virus-infected bacteria.[11]

Biological Effects on Strains of E. coli.—We have described methods for the analysis of the effects of various compounds on bacterial growth, viability, and nucleic acid biosynthesis.[2] Fluorouracil (FU), fluorouracil riboside (FUR), and fluorouracil deoxyriboside (FUDR)[12] were tested for their effects on these functions in various strains of *E. coli*.

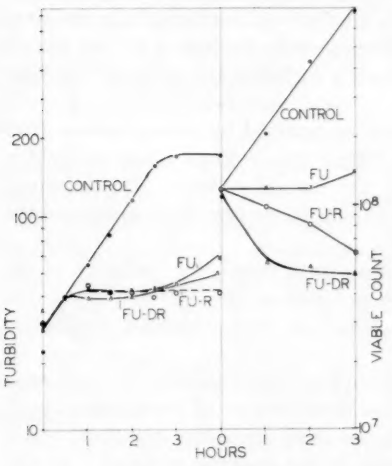

FIG. 1.—The effect of 5-fluorouracil (FU), fluorouracil riboside (FUR), and fluorouracil deoxyriboside (FUDR) on the growth and multiplication of *E. coli* strain B in a glucose-mineral medium. Compounds were present at 0.089 μmole/ml.

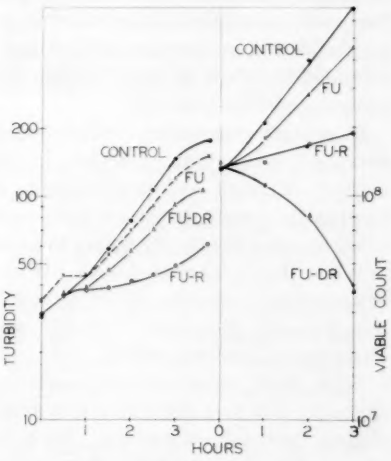

FIG. 2.—The effect of fluorouracil and its nucleosides (0.089 μmoles/ml) on the growth and multiplication of a uracil-requiring strain of *E. coli*.

These compounds had different effects, depending on the compound and strain used. Comparing the three compounds on *E. coli*. strain B, at a concentration of 0.089 μmole/ml., it can be seen in Figure 1 that in each case there is an initial increase of approximately 50 per cent in turbidity, which occurs at the rate observed in the uninhibited culture. This growth then ceases more or less abruptly, although the restraint imposed by FU tends to be overcome after 2 hours. However, the effect on viability can be seen to be quite different in each case. FU produces a

bacteriostasis and occasionally a weak killing effect, while FUR is slowly but markedly bactericidal. FUDR, however, is very rapidly bactericidal, producing a rapid death of about 50 per cent of the cells and is thereafter bacteriostatic. As will be seen below, it appears likely that the initial effect of FUDR arises from the inactivation of thymidylate synthetase by conversion of FUDR to nucleotide, whereas the later bacteriostatic effect in surviving organisms probably arises from the cleavage of FUDR to form free FU.

Studies of nucleic acid biosynthesis in strain B revealed that all three compounds completely inhibited DNA synthesis but permitted at least a doubling of RNA. FU was slightly less inhibitory toward RNA synthesis than were the nucleosides. Thus FU and its derivatives appear to provoke a chemical pattern of biosynthesis and unbalanced growth in strain B comparable to that in thymineless strains lacking thymine, i.e., one in which protein and RNA synthesis continue initially, while DNA synthesis is entirely stopped.

In the uracil-requiring mutant,[13] *E. coli* strain B_{U^-}, different patterns of effects are observed, as presented in Figure 2, when both inhibitory compound and exogenous uracil are present in the medium. Thus only a slight inhibition of turbidity and viable count are obtained in the presence of FU. On the other hand, FUR has the most profound inhibitory effect on growth, an effect which substantially accounts for the slowed increase in viable count. However, with this strain FUDR has an intermediate effect on the inhibition of increase in turbidity but produces the most pronounced killing effect.

In routine experiments, viable counts were determined by colony counts after overnight incubation. Cultures in which killing was evident plate to yield a mixture of small and large colonies unlike the normal cultures. On continued incubation, a small increase in viable count of about 10 per cent is obtained, and the small colonies eventually attain full size.

When B_{U^-} is incubated with FU in the absence of uracil, the turbidity of the culture almost doubles, while almost 80–90 per cent of the cells are killed. In this situation it appears that FU replaces uracil in some functions, facilitating death by unbalanced growth.

With the thymine-requiring bacterium, strain 15_{T^-}, incubated in the absence of thymine, FU and its derivatives inhibit the development of thymineless death slightly but not completely. With the thymineless uracil-less organism, strain $15_{T^-U^-}$, FU does not inhibit thymineless death in the presence of uracil. In this organism also the maintenance and slow increase of viable cells by thymine (0.016 μmole/ml) in the absence of uracil is not affected by FU (0.089 μ mole/ml).

Reversal of Inhibition.—When an inhibitory concentration of FU was added (0.089 μmole/ml) to a growing culture of strain B in the presence of thymine or uracil or both, it was observed that thymine at 0.089 μmole/ml reversed the bactericidal action of the compound but could not markedly stimulate growth. On the other hand, uracil at 0.089 μmole/ml supported almost normal growth. Uracil was used at the concentration which supports the multiplication of strains B_{U^-}; thymine was in fivefold excess. These results are presented in Figure 3. Thus strain B in the presence of FU and uracil behaves similarly to strain B_{U^-} under these conditions.

However, in the presence of FUDR the pyrimidines show different activities, as

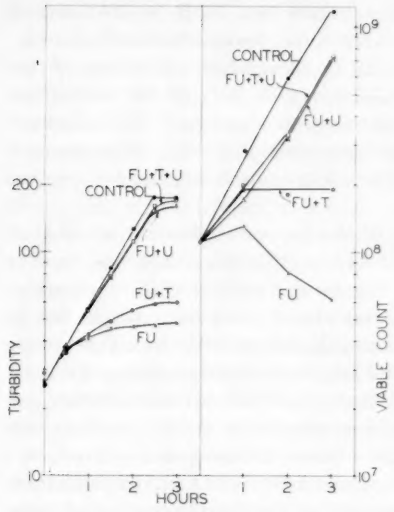

FIG. 3.—The inhibition of *E. coli* strain B by fluorouracil (0.089 μmole/ml) and its reversal by uracil (U) (0.089 μmole/ml) or thymine (T) (0.089 μmole/ml) or both together.

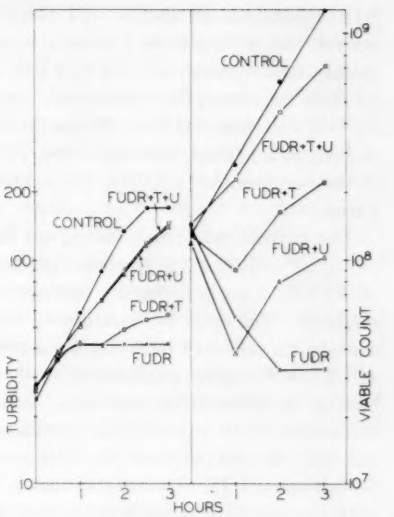

FIG. 4.—The inhibition of *E. coli* strain B by fluorouracil deoxyriboside and its reversal by uracil or thymine or both together.

presented in Figure 4. Thymine, while not supporting very extensive growth, preserves viability. The reverse pattern occurs with uracil, i.e., this pyrimidine permits a rapid and extensive increase in turbidity but in the first hour, at least, actually increases killing. A combination of thymine and uracil are complementary and permit almost normal growth.

Effects on Virus-Infected E. Coli.—FU and its nucleosides are profoundly in hibitory to DNA synthesis in *E. coli* infected with the T-even phages. When strain B_U- is multiply infected by T6r+ in the presence of uracil, there is a tripling of DNA in an hour. FU prevents any increase in DNA in the presence or absence of uracil.

When strain B was infected by T2r+ in the presence of FU or either of its nucleosides, DNA synthesis was completely inhibited. It was found that uracil was ineffective in overcoming the inhibition. However, exogenous thymine partially overcame this inhibition, and added uracil did not augment this reversing action. Comparable reversal patterns were obtained when FU, FUR, or FUDR were used as inhibitors.

When strain B_T- or strain 15_T- was infected with T2r+ in the absence of exogenous thymine, these infected cells became capable of synthesizing DNA and thymine.[11] FUDR totally inhibited such DNA synthesis. In these cases exogenous thymine permitted a restoration to 50 per cent of the maximal rate.

Biochemical Aspects of FUDR Metabolism.—Since the biosynthesis of thymine occurs at the nucleotide level via the conversion of deoxyuridylic acid to thymidylic acid, it appeared reasonable to suppose that the most potent inducer of a thymine requirement, FUDR, was inhibitory after phosphorylation to form 5-fluorouracil

deoxyriboside-5′-phosphate (FUDRP). When strain 15_{T^-} at 5×10^8 bacteria per ml. was incubated in a mineral medium plus glucose, the cells removed approximately 0.045 μmole/ml of FU, FUR, or FUDR in 30 minutes. A culture of 500 ml. was incubated for 30 minutes with 30.5 μmoles of FUDR; 60 per cent of the FUDR was removed from the medium and taken into the bacteria. The cells were chilled, sedimented, and extracted with 2 per cent perchloric acid. After removal of the perchlorate as $KClO_4$, the extract was found to contain 36 μmoles of organic phosphate.

The extract was fractionated on the ion exchange resin, Dowex-1-acetate, at pH 4.2.[14] Nucleotide fractions possessing absorption maxima comparable to that of FUDR were purified by adsorption on Norite and elution with ammoniacal ethanol. The first FU-containing fraction was eluted from the resin at 0.2 *M* acetate, an expected behavior for a pyrimidine nucleotide with the ionizable groups of FUDRP. After purification on Norite, 0.2 μmole of a compound was obtained having an absorption spectrum similar to that of FUDR. This material was shown to inhibit thymidylate synthetase but was completely inactive with respect to the deoxycytidylate hydroxymethylase. Since 5-fluorouracil riboside-5′-phosphate (FURP) has been shown to be completely inactive at high concentrations with thymidylate synthetase, it may be inferred that this fraction contained some FUDRP. As will be seen below, this compound is enormously inhibitory to thymidylate synthetase.

A compound possessing an apparent P to FU content of 2:1 was isolated in an amount of 0.4 μmole following elution at 1.0 *M* acetate. After removal of one P by acid hydrolysis, the compound migrated on paper as did FURP[15] in a solvent containing sodium tetraborate.[16] It showed no trace of FUDRP, as compared with this material synthesized chemically by Farkas *et. al.*[16a] or that synthesized enzymatically in this laboratory. It appeared, therefore, that this isolated compound was the fluorouracilriboside diphosphate.

Small amounts of a fluorouracil compound (*ca.* 1–2 μmoles) was also eluted at 1.5 *M* acetate but was not cleanly separated from another compound possessing an absorption maximum at about 260 mμ. This FU-containing fraction also gave rise to FURP on acid hydrolysis.

It appears, therefore, that far more FUDR is removed from the medium (*ca.* 18 μmoles) by strain 15_{T^-} than can be accounted for in the acid-soluble fraction (a maximum of 3 μmoles). Significant quantities of free FU or of non-phosphorylated derivatives were not found in this fraction. A very small amount of nucleotide FU, i.e., a maximum of 10 per cent, is found in FUDRP, while the remainder appears as ribotides at di- and possibly triphosphate levels. It seems reasonable to suppose that the assimilated FU after conversion to ribotides is incorporated into RNA, as has been reported by other workers.

The Enzymatic Synthesis of FUDRP.—FUDR was incubated with ATP and a partially purified thymidine kinase[17] derived from *E. coli* strain B. The reaction mixture (75 ml.) contained enzyme derived from 6.6 gm. bacteria (wet weight), 400 μmoles Na_4ATP, 32 μmoles FUDR, 200 μmoles $MgCl_2$, and 3,750 μmoles glycylglycine at pH 7.4. After 3 hours at 37° the reaction was stopped by boiling. After centrifugation, the supernatant fluid was concentrated and boiled at pH 1 for 1 hour. The hydrolyzate was concentrated to 2 ml. and passed through a Dowex-

50-H^+ column, which was washed with 0.01 *N* HCl. Only 10 per cent of the original ultraviolet absorption filtered through, and this filtrate was neutralized and fractionated on a Dowex-1-Cl^- column. FU and FUDR were eluted initially by 0.001 *M* HCl, followed by small amounts of adenosine and adenosine-5′-phosphate. FUDRP and some contaminating materials were eluted by 0.05 *M* HCl. A fraction rich in FU was chromatographed on paper in isobutyrate-NH_3 at pH 3.6, and a band (of R_f 0.35) identical with that of synthetic FUDRP was obtained. This material was eluted in a yield of 0.9 μmole and possessed an ultraviolet spectrum with a maximum in 0.1 *N* HCl at 268 mμ. Ratios of the optical densities at wave lengths 250, 260, 270, and 280 mμ were within 10 per cent of the values for synthetic FUDRP. The R_f's of the synthetic and biosynthetic FUDRP also agreed in the ethanol–ammonium acetate–borate system (R_f–0.17) which completely separated the deoxyribotide and ribotide (R_f of FURP was 0.08).

It may be noted that synthetic FUDRP was completely dephosphorylated by the venom of *Crotalus adamanteus*, which was capable of dephosphorylating 5′-nucleotides, but not 2′- or 3′-nucleotides. It was concluded, therefore, that the synthetic material was entirely in the form of the 5′-nucleotide, as was the biosynthetically prepared FUDRP. As will be seen below, both preparations of FUDRP had essentially identical inhibitory activities against thymidylate synthetase.

FUDRP Inhibition of Thymidylate Synthetase.—Thymidylate synthetase and the deoxycytidylate hydroxymethylase are prepared most conveniently from *E. coli* infected with the T-even bacteriophages.[18] Infection of strain B increases the amount of thymidylate synthetase seven- or eight fold over that present in the uninfected cell. Deoxycytidylate hydroxymethylase is absent in extracts prepared from uninfected cells; in extracts from infected cells the amount of this enzyme is about threefold greater than the amount of thymidylate synthetase.

The two enzymes may be separated by salt precipitation; however, the deoxycytidylate hydroxymethylase appears to be considerably more stable following this procedure. Hence it has been necessary at present to test the effect of FUDRP on the thymidylate synthetase in relatively unpurified systems.

The assay for both enzymes relies on the acid-stability of $HC^{14}HO$ fixed to the appropriate pyrimidine nucleotide, and substantially the same system is used in both assays. In unpurified systems of relatively low specific enzyme activity, blank values i.e., the fixation of $HC^{14}HO$ in the absence of pyrimidine nucleotide, are appreciable. In order to increase the sensitivity of the assay for thymidylate synthetase in unfractionated extracts, $HC^{14}HO$ of relatively high specific activity (79,000 c.p.m. per μmole) is used, as contrasted with $HC^{14}HO$ of 22,600 c.p.m. per μmole in the assay for deoxycytidylate hydroxymethylase. The incubation mixture contains in 0.5 ml.: 2.5 μmole of deoxycytidylate or 1.0 μmole of deoxyuridylate, 2.5 μmoles $HC^{14}HO$, 0.5 μmoles tetrahydrofolic acid (THFA), 5.0 μmoles $MgSO_4$, 20.0 μmoles phosphate buffer pH 7.0, and 0.1 or 0.2 ml. enzyme. The system is incubated 20 minutes at 37° and precipitated with 10 per cent TCA. The supernatant fluid (0.5 ml.) is boiled with 0.5 *N* HCl containing 1 per cent $FeCl_3$ to destroy the HCHO-THFA complex. An aliquot of this solution (0.05 ml.) is evaporated on a copper planchet with 0.05 ml. *N* HCl, and radioactivity is measured. Under these conditions, proportionality is obtained as a function of enzyme concentration in the fixation of 0.01–0.05 mole $HC^{14}HO$.

In experiments with FU mononucleotide isolated from the acid-soluble extract of the culture of strain $15T^-$ which had assimilated FUDR, an extract of T6r+-infected *E. coli* strain B (0.5 ml. = 1.1×10^9 bacteria) was preincubated with 0.1 μmole of FU mononucleotide in 0.5 ml. of 0.05 *M* phosphate buffer at pH 7.0 for 15 minutes at room temperature. Equivalent aliquots of a mixture of enzyme and compound and of enzyme alone were compared in the assays presented above. In a 20-minute period, 0.05 ml. of the extract fixed 0.019 and 0.059 μmole of $HC^{14}HO$ with deoxyuridylate and deoxycytidylate, respectively. In this interval, 0.1 ml. of preincubated extract plus inhibitor fixed 0.000 and 0.063 μmoles of $HC^{14}HO$, respectively. Thus the isolated natural product was a specific inhibitor of thymidylate synthetase and did not inhibit the deoxycytidylate hydroxymethylase.

This result has been obtained in subsequent studies with synthetic and biosynthetic FUDRP, using a variety of partially purified enzyme preparations. However, in these tests it was observed that when FURDP was added to the assay system before the addition of enzyme, which was the last component added, inhibition was not complete, particularly with relatively large amounts of enzyme. However, preincubation of enzyme with FUDRP obliterated enzyme activity.

Synthetic and biosynthetic FUDRP were compared in assays in which varying amounts of inhibitor were preincubated with enzyme for 5 minutes at 25° in 0.04 *M* phosphate buffer. An amount of enzyme was used sufficient to fix 0.031 μmole $HC^{14}HO$ in 20 minutes. More than 90 per cent inhibition was obtained at concentrations greater than 0.001 μmole of each type of FUDRP. With 0.001 μmole of FUDRP, i.e., a substrate to inhibitor ratio of 1,000, inhibitions of 35–55 per cent were obtained. Thus the two materials were comparable in inhibitory activity. No inhibition was observed at 0.0005 μmole of FUDRP in this system.

Preincubation of enzyme with deoxyuridylate (1 μmole) could partially protect against the effect of subsequent addition of 0.01 μmole of FUDRP admixed with the other reagents. Pre-incubation of enzyme with HCHO and tetrahydrofolic acid under regular assay conditions also protected slightly against a subsequent addition of 0.005 μmole of FUDRP.

FURP was essentially inactive in these systems at 500 times the concentration at which inhibitory effects could be detected for FUDRP. FU was similarly inactive. On the other hand, FUDR was slightly inhibitory (up to 30–40 per cent) at concentrations of 2 μmoles in 0.5-ml. assay systems.

Discussion.—It appears that FU and FU derivatives affect uracil metabolism in growing bacteria. Thus in strain B the inhibition of growth is largely reversed by the presence of exogenous uracil. This result is consistent with the incorporation of FU into RNA, noted earlier. This phenomenon also seems to have occurred with strain 15_{T^-}. It is possibly significant, however, that the inhibitory effects of FU and FUDR in T2-infected *E. coli* are in no respect reversed by uracil. This perhaps suggests that in these systems RNA metabolism is either unaffected or is not of critical importance.

However, in growing bacteria the effects of FU which may be reversed by uracil are not bactericidal, in contrast to the effects of thymine in reversing the bactericidal actions of FU and FUDR, as shown in the figures. It may be suggested that effects of FU compounds on viability, which are reversible by thymine, are of greater significance from the point of view of the future survival of the organism. It

seems probable, then, that the activities of these compounds in promoting unbalanced growth and thymineless death are of greatest significance in explaining the antitumor activities of FU and FUDR. This question may be readily explored by attempting to reverse the antitumor activities of FU and FUDR by uracil or thymine or by combinations of these pyrimidines. This problem is of great importance in orienting attempts to synergize the activities of these inhibitors by means of uracil analogues or thymine analogues or both, or by still other types of agents.

The provocation of thymine deficiency by FU or FUDR is explainable solely by the inhibitory properties of FUDRP and not by FURP, which is apparently formed in far greater amount, at least in strain 15_{T^-}. FUDRP is a very potent inhibitor of thymidylate synthetase, since it combines irreversibly with this enzyme. Such an effect explains the bactericidal action of the compound, in terms of the critical effects of thymine deficiency in the life of an organism.

Since FUDR is so readily cleaved by a nucleoside phosphorylase, which thereby reduces the amount of compound available for conversion to the true inhibitor, FUDRP, it would evidently be important to explore the possibility of inhibiting this enzyme during tumor chemotherapy. The provision of another source of exchangeable deoxyribose, such as hypoxanthine deoxyriboside, might also be useful in minimizing the action of the phosphorylase. Possibly the most effective approach to this problem would be to devise a method for the penetration of FUDRP itself or of a suitably modified derivative. The use of FUDRP as an inhibitor would avoid the action of nucleoside phosphorylases and minimize the need for a nucleoside kinase, which in *E. coli* at least is not very active.

Summary.—The inhibitory actions of 5-fluorouracil and its nucleosides on growth and multiplication have been studied in *E. coli* and a series of pyrimidine-requiring strains of this organism. Effects have been noted on both uracil and thymine metabolism; those related to uracil requirements inhibit growth but not survival, while effects related to the creation of a thymine deficiency are markedly bactericidal. Of the compounds tested, fluorouracil deoxyriboside is maximally potent in its bactericidal activity.

The deoxyriboside is assimilated by bacteria and is converted in small part to the deoxyribotide, which has been isolated. Fluorouracil deoxyribotide has also been synthesized enzymatically from fluorouracil deoxyriboside to yield a product identical with the synthetic 5′-dexoyribotide. This nucleotide is a highly potent irreversible inhibitor of thymidylate synthetase. This effect explains the capability of fluorouracil and its deoxyriboside to create thymine deficiency, a deficiency which provokes unbalanced growth and cell death.

* These studies have been aided by grants from the Commonwealth Fund and the Upjohn Company.

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[11] The work of this laboratory on thymine metabolism began in 1952 as a result of the study of the biosynthesis of the viral-specific pyrimidine, 5-hydroxymethyl cytosine (HMC). HMC proved not to be a precursor of thymine in the thymine-requiring mutant, *E. coli* strain 15_T-. However, it was observed that infection of this strain with T2 bacteriophage induced the organism, in some manner, to synthesize both thymine and HMC (H. D. Barner and S. S. Cohen, *J. Bact.*, **68**, 80, 1954). It has now been shown in this laboratory that the syntheses of these pyrimidines occur at the nucleotide levels by means of two different enzymes. Thymidylate synthetase: UDRP + HCHO + 2H $\xrightarrow{\text{THFA}}$ TDRP. Deoxycytidylate hydroxymethylase: CDRP + HCHO $\xrightarrow{\text{THFA}}$ HMCDRP.

Infection of *E. coli* strain B by a T-even bacteriophage induces the synthesis of both enzymes, the unique deoxycytidylate hydroxymethylase and thymidylate synthetase. This phenomenon has now been shown to occur as well in the two thymine-requiring strains of *E. coli*, strain 15_T- and strain B_T-. Neither of these organisms contains appreciable base levels of these enzymes in cell-free extracts; however, infection induces the appearance of large amounts of these enzymes.

[12] These compounds were kindly supplied by Dr. R. Duschinsky of Hoffman-LaRoche, Inc., Nutley, N. J. In addition, with the participation of Mr. A. Balsam and Mr. E. Kean, we have synthesized the deoxyriboside in gram quantities by exchanging fluorouracil with thymidine in a ratio of 3 moles of FU to 1 mole of TDR in the presence of a bacterial nucleoside phosphorylase (M. Green and S. S. Cohen, *J. Biol. Chem.*, **225**, 397, 1957). The reaction could readily be followed by paper electrophoresis of the incubation mixture for 2. 5 hours in 0.2 *M* borate buffer at pH 9.2 at 11.4 volts/cm. Thymine and thymidine did not migrate under these conditions, while FU and FUDR migrated readily as anions, the latter somewhat more slowly. The relative amounts of these compounds were estimated by elution of the fluorescence-quenching areas and spectrophotometric analysis of the eluates. In several experiments 32–48 per cent production of FUDR from thymidine were observed. The isolation of FUDR was effected as described by R. Duschinsky, E. Pleven, J. Malbica, and C. Heidelberger, *Absts. Am. Chem. Soc. Meeting, Sept. 1957*, p. 19c. FUDR and FU were separated from thymine and thymidine in a deproteinized system on an ion exchange resin, Dowex-1-formate. Thymine and thymidine were eluted rapidly at pH 9.9, while FUDR and FU were eluted later at pH 7.4. The FU compounds were lyophilized to remove ammonium formate and were finally separated on a cellulose column, using as solvent the non-aqueous layer of a mixture of 65 parts of ethyl acetate to 35 parts water to 5 parts of formic acid (88 percent). FUDR was recrystallized from ethanol petroleum ether.

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[15] A sample of enzymatically synthesized FURP was kindly given to us by Dr. Charles Heidelberger, of the University of Wisconsin.

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[17] The method of isolation of the enzyme was kindly given to us prior to publication by Dr. Arthur Kornberg of Washington University, St. Louis. Purification from the bacterial extract involved streptomycin precipitation of nucleic acid, heat denaturation at 55–60°, and fractional ethanol precipitation of the enzyme.

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CREVICE STRUCTURES IN HEMOPROTEIN REACTIONS

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The hemoproteins are such versatile oxidation-reduction catalysts and such remarkable oxygen-binding pigments that there has always been a lively interest in the structural factors that determine the reactivity of the heme iron atom. The high specificity which characterizes many of their reactions, which is shown in only a rudimentary manner by heme itself, would appear to rule out structures with no direct Fe-protein bonding; so, in general, the binding of the heme can be considered in terms of one or two Fe-protein bonds, with additional bonding through the vinyl and propionic acid side chains. Furthermore, the configuration of the heme is also an important factor. A hemoprotein with the heme bound flat on the surface of the protein would be expected to show differences in reactivity, especially in the case of complex formation with ligands, from one in which the heme is buried in a crevice or fold in the polypeptide chain.

In the past the need to consider the influence of structural factors on reactivity, notably crevice structures, has arisen only in studies on cytochrome-c, hemoglobin, and myoglobin. However. in the present paper it will be shown that certain features of the formation of ferriperoxidase and ferricatalase complexes, which set them apart from the other three hemoproteins, can be explained by the simple hypothesis that a crevice structure with two Fe-protein bonds exists in the parent compounds, and, when a complex formation occurs, one of the bonds is broken. liberating a group with a high proton affinity.[1]

A. STATEMENT OF THE PROBLEM

The fact that ferrohemoproteins form complexes preferentially with neutral ligands such as O_2, CO, NO, and isocyanides, whereas ferrihemoproteins react preferentially with "fluoride," "cyanide," "azide," etc., is very suggestive that in the ferric complexes the anions F^-, CN^-, and N_3^- are bonded to the iron and not the neutral, i.e., uncharged, conjugate acid species HF, HCN, and HN_3.[2] Moreover, this distinction would be in accord with many other reactions in co-ordination chemistry in which fluoride, cyanide, and azide react with transition metal ions.[3] It is not surprising, therefore, that there are great similarities between the magnetic susceptibilities and the visible and near-ultraviolet spectra of the corresponding complexes of ferrihemoglobin, ferrimyoglobin, and ferriperoxidase with fluoride, cyanide, and azide—similarities which extend further to the fluoride and cyanide complexes of ferricatalase.[4, 5] The assumption has accordingly been made and generally accepted that these particular complexes all contain the anions bonded to the iron.

But, in spite of these similarities, the pH variations of the equilibrium constants for complex formation are very different. With relatively strong acid ligand systems, the equilibrium constants for ferrimyoglobin and ferrihemoglobin are approximately independent of $[H^+]$ in the pH range 5–8 and 5–7, whereas those for ferriperoxidase and ferricatalase are directly proportional to $[H^+]$ (see Fig. 1, *A* and

FIG. 1

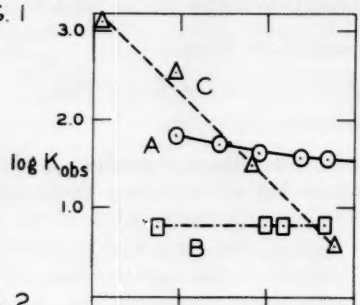


FIG. 2

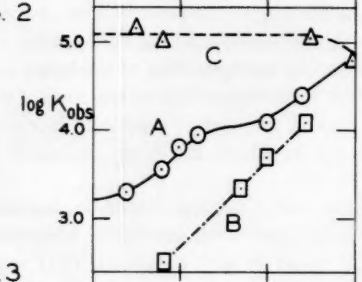
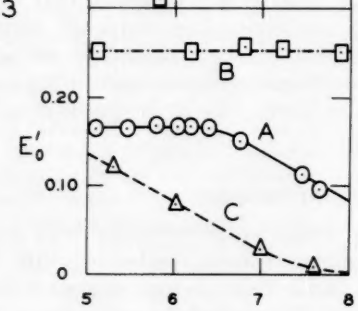


FIG. 3



FIGS. 1–3.—Typical pH variation of log K_{obs} for complex formation by the ferrihemoproteins, and of E_0' for the ferrihemoprotein–ferrohemoprotein couples. For convenience in plotting the data, 1.0 has been subtracted from all the original log K_{obs} values in Fig. 2, A, and 0.3 volts added to all the original E_0' values in Fig. 3, C. FIG. 1: Relatively strong acid ligand systems. *A*, ferrimyoglobin + fluoride,[6] $pK_{HF} = 3.2$; *B*, ferricytochrome-c + Azide,[11] $pK_{HN_3} = 4.7$ *C*, ferriperoxidase + Fluoride.[8] FIG. 2: Very weak ligand systems. *A*, Ferrimyoglobin + cyanide,[6] $pK_{HCN} = 9.5$; *B*, ferricytochrome-c + cyanide,[12] *C*, ferricatalase + cyanide.[10] FIG. 3: E_0' values with respect to the standard hydrogen electrode. *A*, hemoglobin;[14] *B*, cytochrome-c;[15] *C*, peroxidase.[16] Experimental details—ionic strengths, temperatures, etc.—may be found in the references.

C); with very weak acid ligand systems, over the same pH ranges, the equilibrium constants are approximately inversely proportional to $[H^+]$ and independent of $[H^+]$, respectively (see Fig. 2, *A* and *C*).[6–10] The variations for ferricytochrome-c follow those for ferrimyoglobin and ferrihemoglobin with both types of ligand systems (see Figs. 1, *B*, and 2, *B*).[11, 12] These data indicate that a proton participates as a reactant in the ferriperoxidase and ferricatalase equilibria, but not in those of the other hemoproteins over the same pH range.

The pH variations of the oxidation-reduction potentials can be considered in the same context, because a one-equivalent reduction, like complex formation with an anionic ligand, reduces the charge on a metal ion complex by one unit. These pH variations also fall into two classes (see Fig. 3, *A*, *B*, and *C*). Those for myoglobin, hemoglobin, and cytochrome-c are all approximately independent of $[H^+]$ in the pH range 5–7, and correspond to that expected for single-electron transfer,[13–15] i.e.,

$$Fe^{III} + e^- \longleftrightarrow Fe^{II}$$

But the variation for peroxidase involves a direct proportionality to $[H^+]$, indicating that a proton again participates,[16] i.e.,

$$Fe^{III} + e^- + [H^+] \longleftrightarrow Fe^{II}$$

Oxidation reduction between the ferrous and the ferric states and the formation of the ferric complexes thus show entirely consistent behavior.

The following conclusions can be drawn: First, there is some fundamental difference in the composition and structure of either the parent compounds or their complexes, whereby, if anions are bonded to the heme

iron, a proton is bonded elsewhere in the complexes of ferriperoxidase and ferricatalase but not in those of ferrimyoglobin, ferrihemoglobin, and ferricytochrome-c. Second, since the oxidation-reduction reactions show the same behavior, the difference originates in the structure of the common reactant, namely, the parent ferrihemoprotein. The problem is thus to explain how the structure of a ferrihemoprotein can influence the pH dependence of its reactions, and this will first be discussed in general terms.

B. CREVICE STRUCTURES AND HEME REACTIVITY

Two types of crevice structure can be envisaged, depending on whether the iron atom is joined to the protein on one or both sides of the porphyrin ring, and, for brevity, they will be referred to as "one-bond" and "two-bond" crevice structures, respectively. The one-bond crevice structure can be regarded as one extreme (Fig. 4, *A*) in a whole series of structures ranging from another extreme in which the heme is held flat on the surface of the protein (Fig. 4, *B*). In these cases it has been

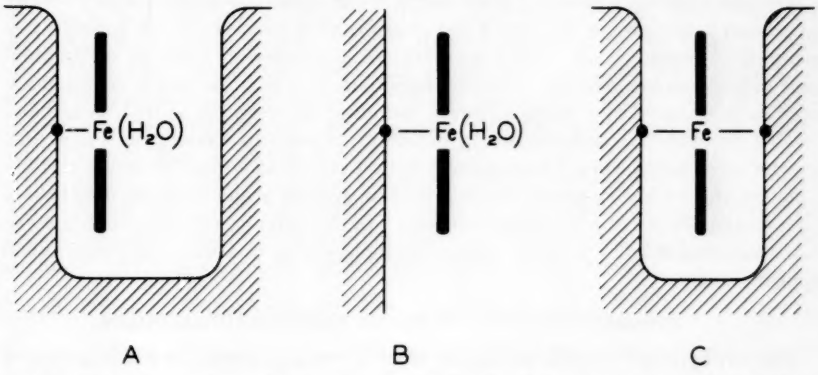


FIG. 4.—Hemoprotein structures. *A*, one-bond crevice; *B*, heme flat on surface; *C*, two-bond crevice. The plane of the porphyrin ring is perpendicular to the paper, and bonding via the side chains has been omitted.

customary to assume that the sixth co-ordination of the iron is occupied by a water molecule. Experiments on the drying of hemoglobin and its derivatives furnish some indirect evidence for the presence of a co-ordinated water molecule of this kind.[17, 18] If, however, the orientation of the heme in the crevice makes possible co-ordination with a second group in the protein to give an intramolecular complex, a two-bond crevice structure is obtained (Fig. 4, *C*).

The one-bond and two-bond crevice structures have an important property in common: the part of the protein which shields the heme may sterically hinder the entry of ligand molecules. It is this aspect of the influence of crevice structures on reactivity that has claimed most attention. Extensive kinetic and equilibrium data for the reaction of cyanide with ferricytochrome-c, which has a well-established two-bond crevice structure[19–22] have been discussed from this point of view.[12] On the basis of equilibrium constants alone, steric hindrance has been suspected in the combination of ferrohemoglobin and ferromyoglobin with *tert*-butyl isocyanide, because the constants are much less than those for the ethyl, *n*-propyl, and *iso*-

propyl derivatives, and, furthermore, no similar decrease is found in the corresponding reactions of ferroheme.[23, 24] A one-bond crevice structure for hemoglobin has also been advanced to explain heme-heme interaction in the oxygenation reaction,[25] and it has been suggested that one heme-linked ionizing group is a histidine residue shielding the heme but held in such a way that direct co-ordination with the iron cannot occur.[26] On the other hand, the very substantial differences between the thermodynamic data for corresponding reactions of ferrihemoglobin and ferrimyoglobin and those of ferricytochrome-c (amounting to 20 kcal/mole and 55 e.u. in ΔH^0 and ΔS^0, respectively) have been taken as evidence that the heme is either flat on the surface in both hemoglobin and myoglobin or else situated in a much shallower crevice than it is in cytochrome-c.[27] In the case of myoglobin in the crystalline state, this conclusion is now amply supported by recent X-ray studies.[28] The heme is held by links to at least four neighboring polypeptide chains, and on one side it is readily accessible from the environment for the attachment of small ligands like oxygen and large ligands such as *para*-iodo-phenylhydroxylamine.

Another important property of all hemoprotein structures and especially crevice structures is the proton affinity of the group that is replaced by the ligand when complex formation occurs. This has attracted little interest, although very significant differences are possible. For hemoproteins in which the sixth co-ordination position is occupied by a water molecule, including, therefore, those with a one-bond crevice structure, the addition of a ligand simply liberates a solvent water molecule. On the other hand, when a hemoprotein with a two-bond crevice structure forms a complex, the weaker Fe-protein bond is broken, and the group liberated may have a high proton affinity or a low proton affinity. In the latter case the reactions may be indistinguishable from those involving the replacement of a water molecule as will appear shortly.

C. HYDROGEN ION PARTICIPATION IN HEMOPROTEIN REACTIONS

The way hydrogen ions are *directly* involved in complex formation will be governed by the following factors, associated with the hemoprotein and with the ligand, respectively:

1. The pK of the group liberated by the ligand (i.e., its proton affinity) relative to the pH of the solution will determine to what extent the addition of a ligand to the iron is accompanied by the addition of a proton to this group.

2. If the ligand participates in an acid-base equilibrium, e.g., F^-, CN^-, etc., then the pK of its conjugate acid relative to the pK of the solution will determine the state of ionization of the uncombined ligand in equilibrium with the complex.

In the absence of other effects, the observed equilibrium constant, calculated in the usual manner from the equation

$$K_{obs} = \frac{[\text{Complex}]}{[\text{Uncombined hemoprotein}][\text{uncombined ligand}]} \quad (1)$$

will show a $[H^+]$ dependence determined solely by the interplay of these factors.

But, in addition, hydrogen ions may be *indirectly* involved through the ionization of heme-linked groups. The resulting effect, first observed in the hemoglobin-oxygen reaction and attributed to groups on the protein in close proximity to the heme, is to change K_{obs} by an amount equivalent to the difference between the free

energy of ionization of the groups in the parent hemoprotein and those in the complex.[29] The pH variation of K_{obs} is, as a consequence, somewhat modified over a limited pH range, but its general character remains unaltered.

Typical reactions of hemoglobin, myoglobin, cytochrome-c, peroxidase, and catalase will now be examined in turn to see how the participation of hydrogen ions, revealed by the pH variations illustrated in Figures 1, 2, and 3, can be accounted for.

a) Hemoglobin and Myoglobin.—On the assumption that ferrihemoglobin and ferrimyoglobin have a structure in which the sixth co-ordination position is occupied by a water molecule, the fluoride and cyanide reactions in the pH range 5–7 can be represented thus:

$$\text{Pr-Fe}^{+}(\text{H}_2\text{O}) + \text{F}^{-} \longleftrightarrow \text{Pr-FeF} + \text{H}_2\text{O} \ldots \quad (2)$$

$$\text{Pr-Fe}^{+}(\text{H}_2\text{O}) + \text{HCN} \longleftrightarrow \text{Pr-FeCN} + \text{H}_2\text{O} + \text{H}^{+} \ldots \quad (3)$$

where Pr stands for the protein moiety and Fe^{+} the protoporphyrin iron atom carrying the net charge of $+1$.[6] The reacting ligands are written as F^{-} and HCN because, with the pK values for HF and HCN of 3.2 and 9.5, respectively, these are the species that predominate in the acid-base equilibria in the pH range 5–7 (see factor 2 above). Inspection shows that equilibrium constants for reactions (2) and (3), calculated using equation (1), will be independent of $[H^{+}]$ and inversely proportional to $[H^{+}]$, respectively, in accord with the main trend of the experimental values (Figs. 1, *A*, and 2, *A*).

In increasingly alkaline solution there is a progressive decrease in the affinity (lower values of K_{obs}) for both ligands. However, the parent hemoproteins show marked changes in color and absorption spectra in these solutions, which have been attributed to the ionization of a proton from the water molecule,

$$\text{Pr-Fe}^{+}(\text{H}_2\text{O}) \overset{K_{Fe}}{\longleftrightarrow} \text{Pr-FeOH} + \text{H}^{+} \ldots \quad (4)$$

with pK_{Fe} having values 8.86 and 9.04 at 20° for ferrihemoglobin and ferrimyoglobin, respectively.[30, 31] This ionization is equivalent to the formation of the hydroxide complex, i.e.,

$$\text{Pr-Fe}^{+}(\text{H}_2\text{O}) + \text{OH}^{-} \longleftrightarrow \text{Pr-FeOH} + \text{H}_2\text{O} \ldots \quad (5)$$

so the decrease in ligand affinity finds a ready explanation in terms of competition between the ligand and OH^{-}. This can be expressed in a slightly different way. In alkaline solution, pH > 10, the ferrihemoproteins are present in the form Pr-FeOH, and reactions (2) and (3) become

$$\text{Pr-FeOH} + \text{F}^{-} + \text{H}^{+} \longleftrightarrow \text{Pr-FeF} + \text{H}_2\text{O} \ldots \quad (6)$$

$$\text{Pr-FeOH} + \text{CN}^{-} + \text{H}^{+} \longleftrightarrow \text{Pr-FeCN} + \text{H}_2\text{O} \ldots \quad (7)$$

On account of its very high proton affinity, the OH^{-} group liberated combines with a proton (see factor 1 above) and, as can be seen from reactions (6) and (7), K_{obs} will be directly proportional to $[H^{+}]$, i.e., it will decrease with increasing pH.

Detailed analysis of the ferrimyoglobin reactions has shown that good quantitative agreement over the whole pH range is obtained if the small, but additional, pH variation is attributed to one heme-linked group.[6] The cyanide reaction (Fig. 2, *A*) shows the effect particularly well. The theoretical equation for the variation

of K_{obs} with $[H^+]$, in terms of the various fundamental constants of the reacting species, is

$$K_{obs} = K_L \cdot \frac{K_{HL}}{(K_{HL} + [H^+])} \cdot \frac{[H^+]}{(K_{Fe} + [H^+])} \cdot \frac{K_r}{(K_r + [H^+])} \cdot \frac{(K_p + [H^+])}{K_p} \cdots \quad (8)$$

where K_{HL}, K_{Fe}, K_r, and K_p are the ionization constants of the ligand conjugate acid HL, and co-ordinated water molecule (reaction [4]), and the heme-linked group in reactant and product, respectively. K_L is the equilibrium constant for the addition of L^- when the heme-linked group is dissociated in both reactant and product, i.e., the parent hemoprotein and the complex.

In ferrohemoglobin and ferromyoglobin it is reasonable to assume that the sixth co-ordination position is also occupied by a water molecule, and the variation of E_0' with pH can be explained in the same way. The theoretical equation, based on the single electron-transfer reaction,

$$\text{Pr-Fe}^+(H_2O) + e^- \longleftrightarrow \text{Pr-Fe}(H_2O) \cdots \quad (9)$$

is analogous to equation (8) with the omission of the term containing K_{HL}. In the case of hemoglobin the onset of the direct proportionality to $[H^+]$ at pH 6.5 (see Fig. 3, *A*) has been attributed to K_{Fe} and K_p having very similar values.[26]

b) Cytochrome-c.—From the time of its discovery, the observation that the absorption spectra of the ferrous and ferric forms of cytochrome-c resemble so closely those of hemochromogens and parahematins left little doubt that the heme is held in a two-bond crevice structure with Fe-N bonds.[19] Later, following on the discussion of heme-linked ionizations in hemoglobin in terms of histidine residues with one joined directly to the iron,[26] the changes in magnetic susceptibility and absorption spectra of ferricytochrome-c over a wide pH range were attributed to the presence of two histidine groups joined directly to the iron.[32] The structure is now known in some detail from amino acid sequence studies on the peptides produced by trypsin digestion.[20-21] It is established that one Fe-histidine bond could be formed within the hemopeptide fragment, which also contains thio-ether linkages binding the heme through α-addition of cysteine SH groups to the vinyl side chains, but the identity of the second group joined to the iron in the native hemoprotein is still an open question.

The pH variations for the ferricytochrome-c reactions throw some light on this problem. Since in this respect ferricytochrome-c resembles ferrihemoglobin and ferrimyoglobin (see Figs. 1, *A* and *B*, 2. *A* and *B*), the reactions must also involve simple replacement:

$$\text{pH} > 6 < 8: \quad \text{Pr-Fe}^+\text{-X} + N_3^- \longleftrightarrow \text{Pr-Fe}N_3\text{X} \cdots \quad (10)$$

$$\text{Pr-Fe}^+\text{-X} + \text{HCN} \longleftrightarrow \text{Pr-FeCN X} + H^+ \cdots \quad (11)$$

where X stands for one of the nitrogenous base groups. It can be seen that the equilibrium constants calculated using equation (1) will be independent of $[H^+]$ and inversely proportional to $[H^+]$, respectively, in accord with experiment. However, if the reactions are to occur in this way, the group X must have a low proton

affinity such that the pH range employed for the equilibrium-constant measurements, beginning at pH 6, lies on the alkaline side of its pK value. Otherwise the addition of a ligand to the iron would be accompanied by the addition of a proton to X. This criterion would appear to exclude all nitrogenous base groups with the exception of the glyoxalinium nitrogen atom of histidine, which, in the free amino acid, has a pK of 6.0.[33] Hence, if the inference based on spectroscopic similarities is correct, one of the groups can be identified as histidine. However, there is no evidence at present to indicate whether it is the one located in the hemopeptide fragment or is a second histidine group situated in a more remote part of the polypeptide chain of the native hemoprotein.

The pH independence of the oxidation-reduction potential follows if the two-bond crevice structure is preserved over the same pH range in ferrocytochrome-c. There is good spectroscopic evidence that this is the case. Reduction then involves single electron transfer:

$$\text{pH 5–7:} \quad \underset{\text{(Ferricytochrome-c)}}{\overline{\text{Pr-Fe}^{+}\text{-X}}} + e^{-} \longleftrightarrow \underset{\text{(Ferrocytochrome-c)}}{\overline{\text{Pr-Fe-X}}}\ldots \qquad (12)$$

It may be noted in passing, however, that data for the cyanide reaction and for oxidation-reduction in more alkaline solution cannot be explained by these reactions alone, and the participation of heme-linked ionizing groups has been suggested.[12, 15]

c) Peroxidase and Catalase.—In the general discussion of crevice structures and heme reactivity, it was envisaged that with two-bond crevice structures the bond broken when complex formation occurs can be to a group having either a low or a high proton affinity; and the structure of cytochrome-c has accordingly been shown to come into the former category. However, the pH variations for the reactions of ferriperoxidase and ferricatalase indicate that these equilibria differ in that a proton participates as a reactant.

This can be accounted for very simply by the hypothesis that ferriperoxidase and ferricatalase have the other kind of two-bond crevice structure where the group liberated has a high proton affinity.[1] If the pH range of equilibrium-constant measurements lies on the acidic side of its pK, addition of the ligand to the iron will be accompanied by the addition of a proton to this group. The fluoride and cyanide reactions can be represented thus:

$$\text{pH 5–7:} \quad \overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{F}^{-} + \text{H}^{+} \longleftrightarrow \overline{\text{Pr-FeF YH}^{+}}\ldots \qquad (13)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HCN} \longleftrightarrow \overline{\text{Pr-FeCN YH}^{+}}\ldots \qquad (14)$$

where the group Y may be either neutral or negatively charged. Inspection shows that the equilibrium constants for reactions (13) and (14), calculated using equation (1), will be directly proportional to $[H^{+}]$ and independent of $[H^{+}]$, as found experimentally (Figs. 1, *C*, and 2, *C*). In the pH range 4–7 the variation of E_0' with pH for ferriperoxidase (Fig. 3, *C*) can be explained in a similar manner by a single equivalent reduction reaction which entails proton addition as well as electron transfer:

$$\text{pH 4–7:} \quad \underset{\text{Ferriperoxidase)}}{\overline{\text{Pr-Fe}^{+}\text{-Y}}} + e^{-} + \text{H}^{+} + \text{H}_2\text{O} \longleftrightarrow \underset{\text{(Ferroperoxidase)}}{\overline{\text{Pr-Fe(H}_2\text{O) YH}^{+}}} \qquad (15)$$

But, as indicated in reaction (15), the further assumption has to be made that, unlike ferrocytochrome-c, the crevice structure is no longer present in ferroperoxidase.

D. EQUATIONS FOR COMPLEX FORMATION WITH TWO-BOND CREVICE STRUCTURES

It will be apparent that the difference between two-bond crevice structures where the groups liberated have high and low proton affinities, respectively, is only one of degree, and the actual pH variation observed might well depend on the pH range of the equilibrium measurements. However, the role which the hemoprotein structure can play is a little more complicated than that described in Section C under factor 1, because a two-bond crevice structure will be stable only over a certain pH range determined by the pK of the group concerned, the strength of the bond to the iron, and the affinity of the iron for OH^-.

In acidic solution, provided that the protein is not denatured and the strength of the crevice bond and the pK of $-YH^+$ have appropriate magnitudes, the crevice will be opened:[34]

$$\overline{\text{Pr-Fe}^+\text{-Y}} + H^+ + H_2O \longleftrightarrow \overline{\text{Pr-Fe}^+(H_2O)\text{YH}^+}\ldots \qquad (16)$$

On the other hand, in alkaline solution, depending on the hydroxyl ion affinity of the iron and, again, on the pK of $-YH^+$, the crevice will be opened:

$$\overline{\text{Pr-Fe}^+\text{-Y}} + H_2O \longleftrightarrow \overline{\text{Pr-FeOH Y}} + H^+\ldots \qquad (17)$$

This is the reaction that corresponds to the ionization of the co-ordinated water molecule with the other type of hemoprotein structure (e.g., ferrihemoglobin and ferrimyoglobin), and it can be formulated as the direct formation of the hydroxide complex in a similar way:

$$\overline{\text{Pr-Fe}^+\text{-Y}} + OH^- \longleftrightarrow \overline{\text{Pr-FeOH Y}}\ldots \qquad (18)$$

Summing up, the crevice structure for the parent hemoprotein will, in principle, be maintained between a pH value in acidic solution, such that $[H^+]$ is no longer high enough for H^+ to compete effectively with Fe^+ for the group Y (reaction [16]), and a pH value in alkaline solution, such that $[H^+]$ is so low that OH^- competes effectively with Y for the Fe^+ (reaction [18]).

Typical reactions representing fluoride and cyanide complex formation and the resulting $[H^+]$ dependence of the equilibrium constants can now be listed for the entire range, from acidic to alkaline solution.

(i) *Acidic Solution.*—Crevice open: uncombined ligands present as HF and HCN:

$$\overline{\text{Pr-Fe}^+(H_2O)\text{ YH}^+} + HF \longleftrightarrow \overline{\text{Pr-FeF YH}^+} + H_2O + H^+\ldots \qquad (19)$$

$$\overline{\text{Pr-Fe}^+(H_2O)\text{ YH}^+} + HCN \longleftrightarrow \overline{\text{Pr-FeCN YH}^+} + H_2O + H^+\ldots \qquad (20)$$

Equilibrium constants for both reactions inversely proportional to $[H^+]$.

(ii) *Less Acidic Solution.*—Crevice intact: uncombined ligands present as HF and HCN:

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HF} \longleftrightarrow \overline{\text{Pr-FeF YH}^{+}}\ldots \quad (21)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HCN} \longleftrightarrow \overline{\text{Pr-FeCN YH}^{+}}\ldots \quad (14)$$

Equilibrium constants for both reactions independent of $[H^+]$.

(iii) *Weakly Acidic Solution.*—Crevice intact: uncombined ligands present as F^- and HCN:

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{F}^- + \text{H}^+ \longleftrightarrow \overline{\text{Pr-FeF YH}^{+}}\ldots \quad (13)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HCN} \longleftrightarrow \overline{\text{Pr-FeCN YH}^{+}}\ldots \quad (14)$$

Equilibrium constants directly proportional to $[H^+]$ and independent of $[H^+]$, respectively.

(iv) *Weakly Alkaline Solution, but pH > pK_{HY}.*—Crevice intact: uncombined ligands present as F^- and HCN:

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{F}^- \longleftrightarrow \overline{\text{Pr-FeF Y}}\ldots \quad (22)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HCN} \longleftrightarrow \overline{\text{Pr-FeCN Y}} + \text{H}^+\ldots \quad (23)$$

Equilibrium constants independent of $[H^+]$ and inversely proportional to $[H^+]$, respectively.

(v) *More Alkaline Solution.*—Crevice intact: uncombined ligands present as F^- and CN^-:

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{F}^- \longleftrightarrow \overline{\text{Pr-FeF Y}}\ldots \quad (24)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{CN}^- \longleftrightarrow \overline{\text{Pr-FeCN Y}}\ldots \quad (25)$$

Equilibrium constants for both reactions independent of $[H^+]$.

(vi) *Alkaline Solution.*—Crevice open: uncombined ligands present as F^- and CN^-:

$$\overline{\text{Pr-FeOH Y}} + \text{F}^- + \text{H}^+ \longleftrightarrow \overline{\text{Pr-FeF Y}} + \text{H}_2\text{O}\ldots \quad (26)$$

$$\overline{\text{Pr-FeOH Y}} + \text{CN}^- + \text{H}^+ \longleftrightarrow \overline{\text{Pr-FeCN Y}} + \text{H}_2\text{O}\ldots \quad (27)$$

Equilibrium constants for both reactions directly proportional to $[H^+]$.

For reactions (i), (iv), (v), and (vi) the equilibrium constants show the same $[H^+]$ dependence as would a hemoprotein with the co-ordinated water molecule structure, but, due to the addition of the proton to Y, the dependence is characteristically different for (ii) and (iii). Other sequences of changes can be obtained, for example, if HF is ionized before $[H^+]$ falls low enough for the crevice to form or if $pK_{HY} > pK_{HCN}$ or if the crevice is opened in alkaline solution at a pH less than pK_{HCN}.

In working out theoretical equations for the variation of K_{obs} with $[H^+]$ over the entire pH range, to cover all these cases, it is necessary to consider, first of all, the equilibrium existing in the absence of any ligand between the intact crevice struc-

ture and an open structure containing the elements of water. This equilibrium will assume one of two forms, depending on whether $-YH^+$ is a stronger or a weaker acid than the co-ordinated water molecule that would otherwise be attached to the iron in the absence of the group Y, i.e.,

$$K_{HY} > K_{Fe}: \quad \overline{Pr\text{-}Fe^+\text{-}Y} + H_2O \underset{K_{cr}}{\longleftrightarrow} \overline{Pr\text{-}Fe^+(H_2O)\ Y} \ldots \tag{28}$$

$$K_{HY} < K_{Fe}: \quad \overline{Pr\text{-}Fe^+\text{-}Y} + H_2O \underset{K_{cr}'}{\longleftrightarrow} \overline{Pr\text{-}FeOH\ YH^+} \ldots \tag{29}$$

For the hemoprotein to exist predominantly in the form $\overline{Pr\text{-}Fe^+\text{-}Y}$ the equilibrium constant for the formation of the crevice bond $Fe^+\text{-}Y$, K_{cr} in reaction (28) or K_{cr}' in reaction (29) must be greater than unity. To evaluate equilibrium constants for complex formation with an added ligand, one particular reacting species now has to be chosen for a reference reaction, and, to facilitate comparison with ferrihemoglobin and ferrimyoglobin,[6] the co-ordinated water molecule structure will again be adopted. As a first approximation it may be assumed that the equilibrium constant K_L is identical for both $\overline{Pr\text{-}Fe^+(H_2O)Y}$ and $\overline{Pr\text{-}Fe^+(H_2O)YH^+}$, and on this basis the two following equations are obtained:

If $K_{HY} > K_{Fe}$,

$$K_{obs} = K_L \times \frac{K_{HL}}{(K_{HL} + [H^+])} \times \frac{[H^+]([H^+] + K_{HY})}{([H^+]^2 + K_{HY}K_{cr}[H^+] + K_{HY}K_{Fe})} \ldots (30)$$

While, if $K_{Fe} > K_{HY}$,

$$K_{obs} = K_L \times \frac{K_{HL}}{(K_{HL} + [H^+])} \times \frac{[H^+]([H^+] + K_{HY})}{([H^+]^2 + K_{Fe}K_{cr}'[H^+] + K_{HY}K_{Fe})} \ldots \tag{31}$$

K_{HL} is the ionization constant of the ligand conjugate acid as before (see eq. [8]).

The change in the $[H^+]$ dependence in acidic solution which is brought about by the closing of the crevice, i.e., from (i) to (ii) in the above sequence of reactions, corresponds to the condition that

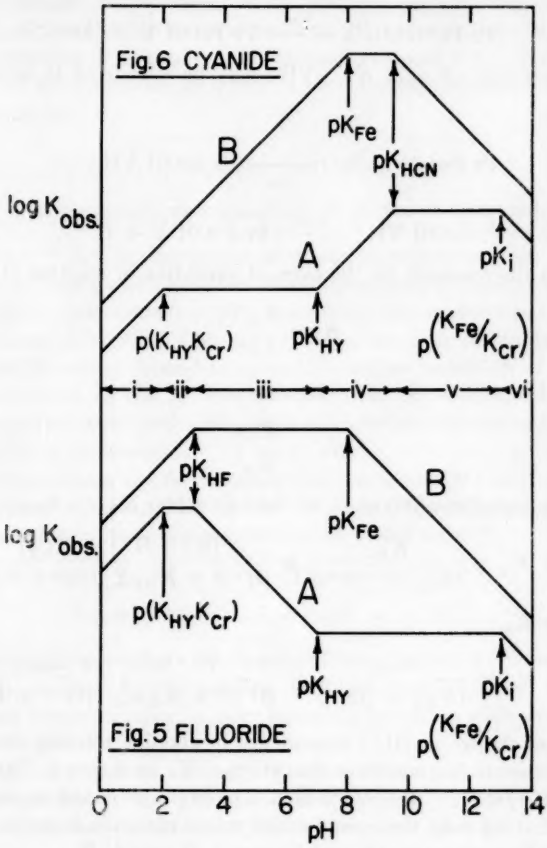
$$[H^+]^2 < K_{HY}K_{cr}[H^+] > K_{HY}K_{Fe}$$

or

$$[H^+]^2 < K_{Fe}K_{cr}'[H^+] > K_{HY}K_{Fe}$$

in the denominator of equation (30) or (31), respectively, while $[H^+]$ remains greater than K_{HY} in the numerator. The change from (iii) to (iv) follows when $[H^+] < K_{HY}$ in the numerator of either equation; and the hemoprotein with the two-bond crevice structure then reacts just like one having only the co-ordinated water molecule. This is illustrated in Figures 5 and 6, where K_{obs} for the formation of fluoride and cyanide complexes of both types of hemoprotein is plotted as a function of pH over the entire range (i)–(vi) according to equations (30) and (8) (omitting any contribution from heme-linked ionizations).

In alkaline solution (vi) it can be seen that the affinity for the ligands again decreases with increasing pH due to competition with the OH^- ion. However, for



FIGS. 5–6.—Schematic plots for the variation of log $K_{obs.}$ with pH for the formation of fluoride and cyanide complexes. Figs. 5, *A*, and 6, *A:* A ferrihemoprotein with a two-bond crevice structure, values chosen for the constants $K_{HY} = 10^{-7}$ $K_{cr} = 10^{6}$, $K_{Fe} = 10^{-8}$. The over-all reactions occurring in the pH ranges (i) to (vi) are listed in Section D. Figs. 5, *B*, and 6, *B:* A ferrihemoprotein with a co-ordinated water molecule structure, value chosen for the constant $K_{Fe} = 10^{-8}$.

the two-bond crevice structure, the change from the "acidic" to the "alkaline" species, corresponding to the formation of the hydroxide complex, is no longer a simple ionization but also involves breaking the crevice (see reactions [17] and [18]). For the case where $K_{HY} > K_{Fe}$, the co-ordinated water molecule may be considered to ionize after reaction (28), i.e.,

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{H}_2\text{O} \underset{K_{cr}}{\longleftrightarrow} \overline{\text{Pr-Fe}^{+}(\text{H}_2\text{O})\ \text{Y}} \ldots \qquad (28)$$

$$\overline{\text{Pr-Fe}^{+}(\text{H}_2\text{O})\ \text{Y}} \xrightleftharpoons{K_{\text{Fe}}} \overline{\text{Pr-FeOH Y}} + \text{H}^{+} \ldots \quad (32)$$

and for the case where $K_{\text{HY}} < K_{\text{Fe}}$, -YH^{+} may be considered to ionize after reaction (29), i.e.,

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{H}_2\text{O} \xrightleftharpoons[K_{cr}]{} \overline{\text{Pr-FeOH YH}^{+}} \ldots \quad (29)$$

$$\overline{\text{Pr-FeOH YH}^{+}} \xrightleftharpoons{K_{\text{HY}}} \overline{\text{Pr-FeOH Y}} + \text{H}^{+} \ldots \quad (33)$$

It follows that the constant for the over-all ionization in reaction (17) will be

$$K_i = \frac{K_{\text{Fe}}}{K_{cr}} \ldots \quad (34)$$

or

$$K_i' = \frac{K_{\text{HY}}}{K_{cr}'} \ldots \quad (35)$$

Substitution in equations (30) and (31) then gives, for $K_{\text{HY}} > K_{\text{Fe}}$,

$$K_{\text{obs}} = K_{\text{L}} \times \frac{K_{\text{HL}}}{(K_{\text{HL}} + [\text{H}^{+}])} \times \frac{[\text{H}^{+}]([\text{H}^{+}] + K_{\text{HY}})}{[\text{H}^{+}]^2 + K_{\text{HY}}K_{cr}([\text{H}^{+}] + K_i)} \ldots \quad (36)$$

and for $K_{\text{Fe}} > K_{\text{HY}}$,

$$K_{\text{obs}} = K_{\text{L}} \times \frac{K_{\text{HL}}}{(K_{\text{HL}} + [\text{H}^{+}])} \times \frac{[\text{H}^{+}]([\text{H}^{+}] + K_{\text{HY}})}{[\text{H}^{+}]^2 + K_{\text{Fe}}K_{cr}'([\text{H}^{+}] + K_i')} \ldots \quad (37)$$

Hence the final change in $[\text{H}^{+}]$ dependence in alkaline solution from (v) to (vi) corresponds either to the condition that $[\text{H}^{+}] < K_i$, as shown in Figures 5, *A*, and 6, *A*, or that $[\text{H}^{+}] < K_i'$. By comparison, a more simple condition obtains with the hemoprotein having only the co-ordinated water molecule, namely, $[\text{H}^{+}] < K_{\text{Fe}}$, as can be seen from equation (8) and Figures 5, *B*, and 6, *B*.

E. FURTHER DISCUSSION OF A TWO-BOND CREVICE STRUCTURE FOR FERRIPEROXIDASE AND FERRICATALASE

There are insufficient data available at present to decide whether the pH variations for the formation of ferriperoxidase and ferricatalase complexes revert to those characteristic of ferrihemoglobin and ferrimyoglobin when measurements are made in rather alkaline solution. Such a change, from (iii) to (iv) in the sequence of reactions in Section D, would furnish substantial evidence for the participation of the bonding group -Y. Nevertheless, the ionization of ferriperoxidase between pH 10.5 and 11.5,[8] which is accompanied by marked changes in absorption spectra and magnetic susceptibility somewhat similar to those observed with ferrihemoglobin and ferrimyoglobin, is itself indicative of a fundamental alteration in the $[\text{H}^{+}]$ dependence. An inspection of the detailed steps involved in the ionization of a two-bond crevice structure, given above in reactions (28) and (32) or (29) and (33), shows that the formation of a hydroxide complex can, in fact, occur only in solution alkaline to the pK of the group -YH^{+}. This can also be seen from the

following comparison. In relatively acidic solution the formation of the fluoride and cyanide complexes of ferriperoxidase show pH variations characteristic of the simultaneous addition of a proton, as in reactions (13) and (14). Now the equivalent reaction for the formation of a hydroxide complex, consistent with these pH variations, would be

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{H}_2\text{O} \longleftrightarrow \overline{\text{Pr-FeOH YH}^{+}} \ldots \qquad \text{(i.e., 29)}$$

But this is not an ionization process, since no proton is liberated. The species formed, $\overline{\text{Pr-FeOH YH}^{+}}$, is simply one of the open structures that, by definition, can be present to only a small extent in equilibrium with the crevice structure, and its concentration is independent of pH; moreover, this reaction has already been taken into account as reaction (29) above. The complete formation of a species containing -FeOH is thus dependent upon the further ionization of $-\text{YH}^{+}$.

The relative merits of the present interpretation of ferriperoxidase and ferricatalase complex formation and three other explanations that have been advanced in the past will now be discussed.

First, if ferriperoxidase and ferricatalase differ structurally from ferrihemoglobin and ferrimyoglobin in having OH^{-} bonded to the iron instead of a water molecule, even in neutral solution, then complex formation would occur:[35]

$$\text{pH 5–8:} \qquad \text{FeOH} + \text{F}^{-} + \text{H}^{+} \longleftrightarrow \text{FeF} + \text{H}_2\text{O} \ldots \qquad (38)$$

$$\text{FeOH} + \text{HCN} \longleftrightarrow \text{FeCN} + \text{H}_2\text{O} \ldots \qquad (39)$$

But this explanation fails to take into account the ionization of ferriperoxidase with pK$\sim$11, which, in view of the similarity with the ionizations that occur in ferrihemoglobin and ferrimyoglobin, is more naturally identified with the formation of the hydroxide complex.

Second, if the four parent ferrihemoproteins all have the co-ordinated water molecule structure but in the peroxidase and catalase complexes the conjugate acids are bonded to the iron instead of the anions, then complex formation would proceed:[10]

$$\text{pH 5–8:} \qquad \text{Fe}^{+}(\text{H}_2\text{O}) + \text{F}^{-} + \text{H}^{+} \longleftrightarrow \text{Fe}^{+}(\text{HF}) + \text{H}_2\text{O} \ldots \qquad (40)$$

$$\text{Fe}^{+}(\text{H}_2\text{O}) + \text{HCN} \longleftrightarrow \text{Fe}^{+}(\text{HCN}) + \text{H}_2\text{O} \ldots \qquad (41)$$

This can be criticized on the grounds that the structures of the complexes differ from those of hemoglobin and myoglobin, which seems contrary to the many similarities in physical properties. Moreover, the protons bound in complexes of this kind would be expected to ionize at some point in the pH range from acidic to alkaline solution. For example, even with ferrocyanic acid, $\text{H}_4\text{Fe(CN)}_6$, the ionization of the last proton, from HFe(CN)_6^{3-}, which might well be regarded as unfavorable on account of the large negative charge, nevertheless occurs with a pK of about 4.[36] Furthermore, the conjugate acids of the simple complexes formed between transition metal ions and fluoride ions, e.g., H_3FeF_6, are strong acids already dissociated in solutions of very low pH.[3]

The third explanation requires the postulate of a heme-linked ionizing group in peroxidase and catalase that changes its acid strength when complex formation

occurs, so that, while it is in the conjugate base form in the parent compounds, it is in the conjugate acid form in the complexes over a pH range extending at least from pH 5 to 8.[37] Denoting the group by HZ-, complex formation can be represented thus:

$$\text{pH 5–8:} \quad Z^-\text{-Pr-Fe}^+(H_2O) + F^- + H^+ \longleftrightarrow HZ\text{-Pr-FeF} + H_2O \ldots \quad (42)$$

$$Z^-\text{-Pr-Fe}^+(H_2O) + HCN \longleftrightarrow HZ\text{-Pr-FeCN} + H_2O \ldots \quad (43)$$

But the increment between the pK of HZ- in the parent compound and in the complex would need to be at least three pH units, far greater than the pK increments obtained for the heme-linked groups in hemoglobin and myoglobin derivatives which range from about 0.3 to 1.2 pH units.[6] The assumption that there are several groups of this kind in peroxidase and catalase with suitably overlapping pK ranges could meet this objection, but it seems very unlikely for structural reasons.

However, there is no very precise and exclusive definition of a heme-linked ionizing group. Sometimes it has been defined on a structural basis as one linked to the iron, and so the co-ordinated water molecule has been included in this category; at other times the "linked" thermodynamic behavior has been emphasized, namely, the way the ligand affinity of the iron depends upon the extent to which the group is ionized.[29] Hence the -YH^+ group in the reactions of the two-bond crevice structure could very well be regarded as a heme-linked group, for not only is it structurally linked to the iron when present on the conjugate base -Y over a certain pH range, but the ionization of -YH^+ has a profound effect on ligand affinity. Furthermore, reference to Figures 5, *A*, and 6, *A*, and equations (30) and (31) shows that the breaking of the bond to Y in acidic solution and the ionization of -YH^+ in more alkaline solution combine to give an apparent "pK increment" which has the value log K_{cr} if $K_{HY} > K_{Fe}$, or log $(K_{cr}'K_{Fe}/K_{HY})$ if $K_{HY} < K_{Fe}$. In principle, this increment can be many pH units, since there is no restriction on the magnitudes of K_{cr}, etc., and the crevice-forming group can thus produce a "heme-linked" effect over a much bigger pH range than would seem possible with the group HZ- in reactions (42) and (43). But -YH^+, by virtue of the direct bonding of Y, cannot undergo a truly independent ionization in the parent hemoprotein, and it therefore differs from the type of heme-linked group believed to be present in hemoglobin and in myoglobin, which is capable of independent ionization in both the parent compound and the complex (see Sec. C, eq. [8], and reactions [42] and [43]). A clear distinction should therefore be made. For a similar reason the co-ordinated water molecule is a different kind of linked group. Although it can ionize in the parent compound, it cannot in the complex, since, having been replaced by the ligand, it is no longer an integral part of the hemoprotein structure. A more detailed comparison of these effects, on the basis of equations (8) and (30), has been given in a recent review.[38]

A major criticism of a two-bond crevice structure for ferriperoxidase and ferricatalase in relation to a co-ordinated water molecule structure for ferrimyoglobin and ferrihemoglobin is that the spectra of all four hemoproteins are very similar and differ significantly from that of ferricytochrome-c. While this is true, it does not necessarily rule out a crevice structure. Similar absorption spectra might arise because the two-bond crevice retains a co-ordinated water molecule, as suggested in another context for hemoglobin,[26] or because some atom other than nitrogen is

directly bonded to the iron. Several complexes are known where the spectra closely resemble that of the parent hemoprotein—for example, cyanate, thiocyanate, formate, acetate, and propionate.[39-41] Alcohols and phenols have been observed to combine with hemin, giving complexes with spectra like those of acidic ferrihemoglobin and ferrimyoglobin;[42] and in alkaline solution phenol, in the form of the phenoxide ion, forms a complex with ferrimyoglobin having a spectrum similar in some respects to that of catalase.[43] Of the amino acids commonly present in hemoproteins, serine, threonine, tyrosine, and possibly hydroxyproline have HO-groups in their side chains and can thus be regarded as potential crevice-forming groups. Moreover, a crevice could be closed, not by an ordinary amino acid side chain of the protein, but by some special group. For instance, in vitamin B_{12} there is the nucleotide co-ordinated to the Co^{III} via an amino-alcohol attached to the propionic acid side chain of the porphyrin-like ring.[44] If there were one special group per iron atom, it would be present in peroxidase and catalase in molecular weight units of 41,000 and 59,000, respectively, and in such low concentrations it might well be very difficult to detect in the protein hydrolyzates.

There is scarcely any evidence at present apart from the pH variation of the equilibrium constants to support the crevice structure; yet it may be significant that ferriperoxidase and ferricatalase are sometimes converted on storage into derivatives having the characteristic absorption spectra of parahematins.[5] This cannot be attributed to gross denaturation, since, on addition of the normal ligands, the familiar complexes are again formed. Similar behavior under such mild conditions has not been reported in the case of ferrihemoglobin and ferrimyoglobin, although with far more drastic treatment, in which the hemoproteins are dried and dehydrated to such an extent that the water-vapor pressure reaches about 12 mm., ferrohemoglobin yields a hemochromogen derivative and ferrihemoglobin a parahematin derivative.[17, 18] There is some evidence to suggest that intermolecular bonding occurs.[45] However, if the conversion of ferriperoxidase and ferricatalase is an intramolecular process, it is easy to visualize how a two-bond crevice structure present initially could facilitate the reaction. All that would be required would be a minor conformational change within the crevice that dislodges the normal group (-Y) and brings a nitrogenous base group into such a position that an Fe-N bond can form.

Although the nature of the Fe-protein bond in hemoglobin has often been discussed and bonding to histidine is regarded as the most likely,[2] there is scarcely any information on hemoglobin or its reactions that provides direct chemical evidence about even the type of group to which the iron is joined. However, it has recently been pointed out that the simplest interpretation of the experiments referred to above substantiates the hypothesis that an Fe-N bond is already present in the native hemoprotein, because, on drying, only the second Fe-N bond would then have to be formed.[38] For the same reason, the conversion of ferriperoxidase and ferricatalase into parahematins is a strong indication that one Fe-N bond is already present in these hemoproteins too. The structural environment of the heme iron atom can be represented thus:

Ferrihemoglobin and ferrimyoglobin: Protein(nitrogenous base)-$Fe^{+}(H_2O)$

Ferriperoxidase and ferricatalase: Protein(nitrogenous base)-Fe^{+}-Y

and its different reactivity would be attributable to the participation of the group Y and the possibility (or likelihood) that the nitrogenous base groups are not the same.

Finally, this kind of structure for ferriperoxidase and ferricatalase would provide a new basis for understanding why they behave differently from ferrihemoglobin and ferrimyoglobin when they react with peroxides and other strong oxidizing agents. The compounds formed, which play the role of "intermediate compounds" if an oxidizable substrate is present and catalytic oxidation ensues, have been shown to be higher oxidation states of the heme group.[46] While ferrimyoglobin and ferrihemoglobin give a single intermediate compound, one oxidation equivalent above the ferric state, ferriperoxidase and ferricatalase give two far more reactive intermediate compounds, one and two equivalents, respectively, above the ferric state. Furthermore, the absorption spectra of the one equivalent higher oxidation states are very different. With a crevice structure in the parent hemoprotein, there would be the possibility that it remained intact in one or in both of these higher oxidation states which are responsible for the highly specific catalytic activity.

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THE SYNTHESIS OF HEMOGLOBIN IN A CELL-FREE SYSTEM

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The microsomal particles[1] have been implicated as the major sites of protein synthesis within the cells of a variety of tissues. The studies of Borsook, Zamecnik, Hultin, and others, with intact animals and various types of whole cell systems (see review by Askonas *et al.*[2]), as well as electron-microscope studies,[3] provided the original evidence for this conclusion. Zamecnik and Keller[4] have, in addition, developed cell-free systems which incorporate C^{14}-labeled amino acids into protein. These include microsomes as well as various soluble enzymes.[5] Further studies have partially defined the intermediate stages involved.[6] However, it has not yet been possible to equate incorporation of labeled amino acid into protein with actual protein synthesis (see review by Campbell[7]).

Kruh and Borsook[8] have demonstrated that rabbit reticulocytes synthesize hemoglobin in vitro and that approximately 85 per cent of the soluble protein made by such cells is of this one species. Studies with whole cells by Rabinovitz and Olson[9] have shown that microsomes participate in the synthesis of hemoglobin.

These authors have also demonstrated incorporation of iron into hemoglobin in a cell-free system containing reticulocyte microsomes.[10]

It is clear, then, that reticulocytes offer a favorable system for study of the synthesis of a specific protein. This paper describes a cell-free system consisting of reticulocyte microsomes and soluble enzymes which possesses the ability to produce hemoglobin.

METHODS AND MATERIALS

Preparation of Microsomes and Enzymes.—Rabbit reticulocytes were prepared and washed as described by Borsook *et al.*[11] All operations were performed at 4° C. unless otherwise indicated. The packed cells (usually 50 ml. from 2 rabbits) were lysed by the addition of 4 volumes of 0.005 *M* magnesium chloride, and the mixture was stirred gently for 10 minutes. One volume of 1.5 *M* sucrose containing 0.15 *M* potassium chloride was added slowly, and the mixture centrifuged at 10,000 × *g* for 10 minutes. The precipitate, containing cell membranes and unbroken cells, was discarded. The solution was then centrifuged for 1 hour at 105,000 × *g*, yielding a precipitate and supernatant (super I). The precipitate (microsomes) was washed by suspending in 100 ml. of Medium A[5] and was centrifuged for 1 hour at 105,000 × *g*. The washed microsomes were suspended in Medium A, yielding a pale-yellow, opalescent solution (20–28 mg. of ribonucleoprotein per ml.). The supernatant (super II) was discarded.

The dark-red supernatant from the first high-speed centrifugation (super I) was adjusted to pH 5.15 with 1 *N* acetic acid, centrifuged, and the precipitated protein dissolved in 0.1 *M* Tris buffer, pH 7.5 (pH 5 enzyme, 12 mg. of protein per ml.). Guinea-pig liver pH 5 enzyme was prepared in a similar way[12] but was reprecipitated at pH 5.15 before use.

Assay Procedure.—The complete reaction mixture contained 0.5 ml. of microsomes; 0.4 ml. of pH 5 enzyme; 150 μg. of creatine kinase purified through the alcohol fractionation;[13] 1 μmole of potassium adenosine triphosphate (ATP), adjusted to pH 7.5 with potassium hydroxide; 20 μmoles of creatine phosphate (CrP), adjusted to pH 7.0 with hydrochloric acid; 0.25 μmole of guanosine triphosphate (GTP); 0.1 μmole of C^{14}-labeled amino acid (leucine, isoleucine, and valine were the uniformly labeled L-isomers obtained from Nuclear-Chicago, Inc., diluted to a specific activity of 3,800 counts/min/mμmole); and the complete amino acid mixture, in a final volume of 1.4 ml. Creatine phosphate was obtained from the California Corporation for Biochemical Research, Los Angeles, California, and other nucleotides were products of the Pabst Laboratories. The complete amino acid mixture contained the 18 amino acids in the proportions, but at one-sixth the final concentration, described by Borsook and co-workers.[14] The C^{14}-labeled amino acid under study was omitted from the mixture of unlabeled amino acids. In some experiments, 0.2 ml. of super I, containing approximately 30 mg. of hemoglobin per ml., was added.

After incubation for the indicated time (15–60 minutes) at 37° C. with shaking, the proteins were precipitated and washed once with 3.5 per cent trichloracetic acid, dissolved in 0.5 ml. of 1 *N* sodium hydroxide, reprecipitated, and washed once with trichloracetic acid, then heated at 60° C. for 10 minutes in 3 ml. of 95 per cent ethanol. Two volumes of ether were added after heating, to insure precipitation

of protein, and the precipitate was finally washed twice with ether. The dried precipitates were weighed and counted with a Nuclear "Micromil" window gas-flow counter. All results are corrected for self-absorption. The results are given as counts per minute (c.p.m.) per milligram of microsomes, based on the actual dry weight of a sample of microsomes washed as described above. This precipitate was found by ribose analysis to contain 30–40 per cent ribonucleic acid (RNA).

Isolation of Hemoglobin.—The large-scale incubation for the isolation of hemoglobin contained (in one beaker) the usual assay constituents at a sixfold level, using reticulocyte pH 5 enzyme, and with 1.2 ml. of super I added. The mixture was incubated for 60 minutes and then cooled in an ice bath. An additional 1.3 ml. of super I was then added, to bring the total carrier hemoglobin to about 60 mg. The mixture was centrifuged for 1 hour in the cold at 105,000 × *g* to remove microsomes. The supernatant proteins contained approximately 103,000 c.p.m. This solution was adjusted to pH 5.15 and the precipitate removed. The supernatant was immediately readjusted to pH 7.0. The proteins precipitated at pH 5 contained 36,000 c.p.m. This precipitate probably includes, in addition to pH 5 enzyme, aggregated ribonucleoprotein from microsomal degradation and absorbed hemoglobin (estimated as 10 per cent of the supernatant hemoglobin). The supernatant solution was dialyzed overnight against 0.1 *M* Tris buffer, pH 7.5, and the hemoglobin then precipitated by addition of solid ammonium sulfate to 90 per cent saturation. The precipitate was dissolved in water and dialyzed against developer No. 2, as described by Allen and co-workers.[15] An aliquot of the dialyzed solution, containing 31,000 c.p.m., was then chromatographed on Amberlite IRC-50 resin, using this same developer. Since rabbit hemoglobin is adsorbed on the resin more strongly than human hemoglobin, the final elution was done at room temperature.[16] The optical densities at 280 and 415 mμ were recorded for each fraction. For determination of radioactivity, the protein was precipitated with trichloracetic acid, followed by the ethanol and ether washes as described above. Carrier hemoglobin was added where needed to provide 10–15 mg. of dry protein per sample for counting.

EXPERIMENTS AND RESULTS

Properties of Incorporation System.—The results in Table 1 show incorporation of C^{14}-labeled leucine into protein, using microsomes from rabbit reticulocytes, plus

TABLE 1

INCORPORATION OF C^{14}-LEUCINE INTO PROTEIN

Reaction Mixture	C.p.m./Mg Microsomes
Complete system,* g. pig pH 5 enzyme	670
Complete system,* minus amino acid mix	366
Complete system,* minus pH 5 enzyme	192
Complete system,* minus pH 5 enzyme minus amino acid mix	110
Complete system,* minus ATP, minus CrP	45
Complete system,* with reticulocyte pH 5 enzyme	806

* The complete system is described in the text. The incubation time was 30 minutes.

pH 5 enzymes from either guinea-pig liver or reticulocytes. The process is energy-dependent, as originally observed by Zamecnik and Keller.[4] No incorporation occurred in the absence of microsomes, with boiled microsomes, or with cell debris substituted for microsomes. Five μg. of crystalline ribonuclease reduced incorpora-

tion more than 90 per cent. Substitution of 10 μmoles of ATP for the CrP inhibited incorporation almost completely. When GTP was omitted, the activity was reduced to half that of the complete system, even without removal of nucleotides.[5] In contrast to preparations of liver microsomes,[17] the results with different batches of reticulocytes were reproducible under standard conditions, agreeing with one another to within 15–20 per cent.

Addition of the complete amino acid mixture developed by Borsook and coworkers[14] doubled the incorporation of C^{14}-leucine, as shown in Table 1, and had a similar effect on C^{14}-valine incorporation in other experiments. This amino acid mixture was designed to yield optimal synthesis of hemoglobin by whole cell suspensions of rabbit reticulocytes. In this respect amino acid "incorporation" in the cell-free system resembles protein synthesis by whole cells.[8] Addition of an amino acid mixture containing each amino acid at a uniform concentration (0.25 μmoles per beaker) was inhibitory.

The finding that only freshly-prepared pH 5 enzyme was effective in stimulating incorporation in the cell-free system suggests that labile enzymes play a role in this stimulation. Studies with guinea-pig liver pH 5 enzyme have shown that certain activating enzymes which are present in very low amounts in fresh preparations are inactive after overnight storage,[18] either at 4° or at −20° C. Thus it is possible that all the activating enzymes are needed for incorporation in the cell-free system, although other labile enzymes may be important also. More detailed investigation of these points is required, however, since the optimal assay conditions have not been studied in detail. For example, the addition of glutathione (5 μmoles per beaker) and extra magnesium ion (3 μmoles per beaker) each increased the incorporation of C^{14}-leucine by 15 per cent and were additive when both were present. The addition of 0.2 ml. of super I increased incorporation sometimes as much as twofold, but this effect varied with different microsome preparations.

Isolation of Radioactive Hemoglobin.—In most cell-free amino acid incorporation systems[4, 17] the reaction ceases after 15–20 minutes of incubation. Progress curves with reticulocyte microsomes (complete system) show an almost constant increase in incorporation for at least 45 minutes and occasionally for 60 minutes. It is probable that amino acids are assembled into protein in the ribonucleoprotein particles and then secondarily released into the cytoplasm as completed proteins.[19] A failure to release completed proteins from the microsomes in other cell-free systems might account for the cessation of the reaction after a short time.

The continued incorporation for longer time periods with reticulocyte microsomes suggested that soluble proteins were being produced. To study this, the usual mixture, after incubation, was centrifuged for 1 hour at 105,000 × g, and microsomes and soluble proteins were washed separately as described above. After a 30-minute incubation time, 10–15 per cent of the radioactivity was present in soluble proteins. When the microsomes from these experiments were frozen and thawed or treated with desoxycholate,[20] the radioactivity in soluble protein was increased only slightly. After 1 hour of incubation, however, 50–60 per cent of the radioactivity was present in soluble proteins. The following experiment demonstrates that the C^{14}-labeled soluble proteins consist mainly of labeled hemoglobin.

A large-scale incubation was carried out as described in "Methods and Materials,"

and the soluble proteins were chromatographed. The coincidence of radioactivity with the hemoglobin peak (Fig. 1) demonstrates that C^{14}-labeled hemoglobin had been formed. Little, if any, non-heme protein was present in the major peak, as shown by the constant ratio of optical density at 415mμ to that at 280mμ throughout the greater portion of the peak. The specific activity (ratio of counts to optical density at 280 mμ) remained constant throughout the hemoglobin peak and was also similar to the specific activity of the mixture of proteins in the first small peak. The recovery of hemoglobin and protein (based on the optical densities) was over 90 per cent of the amount put on the column, including a small protein peak, which was eluted with higher buffer concentrations (not shown in Fig. 1). The recovery of radioactivity was 86 per cent, e.g., 3,000 c.p.m. in the first peak, 21,500 c.p.m. in the hemoglobin peak, and approximately 2,000 c.p.m. with the stronger buffer elution, out of a total of 31,000 c.p.m. originally put on the column. The distribution of radioactivity suggests that all the soluble proteins are being synthesized in the cell-free system. According to the results given above, hemoglobin synthesis accounts for about 82 per cent of the total protein recovered from the column.

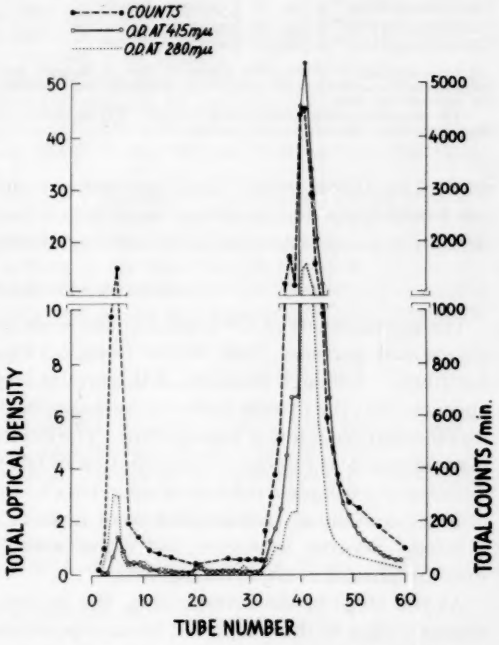

FIG. 1.—Chromatogram of soluble proteins on IRC-50. The column was eluted at 4° until tube 32, and then at room temperature.

Incorporation of Various Amino Acids.—Further information, which indicates that "incorporation" in this system is synonymous with synthesis of normal cell proteins, is provided by the ratios of the amounts of C^{14}-labeled leucine, isoleucine, and valine incorporated. As shown in Table 2, these ratios coincide closely with the proportions of these amino acids in crude globin (the total soluble proteins of the reticulocyte). This agreement is further evidence that these proteins, of which hemoglobin is the largest constituent, are being synthesized. The data also permit a distinction to be made between the "structural" protein of the microsome and the protein being synthesized. Since 50 per cent of the radioactivity remained in the microsomes and the isoleucine and leucine content of microsomal protein are nearly the same,[21] incorporation into microsomal protein would have changed the observed ratios. Therefore, it is probable that the radioactivity in the microsomes largely represents hemoglobin precursors. A similar conclusion has been

TABLE 2

INCORPORATION OF C^{14}-LABELED AMINO ACIDS COMPARED TO AMINO ACID COMPOSITION OF RABBIT GLOBIN

Reaction Mixture	C.p.m./Mg Microsomes	Ratio	Amino Acid Composition†	Ratio
Complete system,* g. pig pH 5 enzyme, leucine	748	1.00	11.7	1.00
Complete system,* g. pig pH 5 enzyme, isoleucine	90	0.12	1.3	0.11
Complete system,* g. pig pH 5 enzyme, valine	538	0.72	8.1	0.77

† From analyses of crude rabbit globin (P. Ts'o, J. Bonner, and H. Dintzis, unpublished data). The amino acid composition is given as gm. per 100 gm. of protein, and the ratios are given as molar ratios for comparison with the radioactivity data.

* The complete system is described in the text. The incubation time was 60 minutes and the total protein of the reaction mixture was washed and counted.

reached by Dintzis *et al.*[22] based on studies with whole reticulocytes. In general, this would imply that the ribonucleoprotein serves as a template for protein synthesis but not as a precursor of the proteins synthesized.[23, 24]

DISCUSSION AND SUMMARY

The incorporation of C^{14}-labeled amino acids into protein in a system containing microsomal particles from rabbit reticulocytes plus soluble enzymes has been described. Although the study of this system is still in its early stages, the evidence indicates that the process under investigation is the synthesis of soluble cell protein which consists mainly of hemoglobin. The evidence which leads to this conclusion is summarized as follows. Incorporation of labeled amino acid was stimulated by addition of a complete mixture of amino acids of a particular composition. Soluble proteins containing radioactivity were formed, and radioactive hemoglobin was isolated. Leucine, isoleucine, and valine were incorporated in the ratios in which they are present in crude hemoglobin.

At this stage of the investigation, the factors required for maximum synthesis appear similar to those reported for incorporation into microsomes of other animal tissues.[7] These findings strengthen the concept of Hoagland *et al.*[25] that the intermediate stages in protein synthesis involve amino acid activation followed by formation of amino acid-RNA.[12, 26] The results also provide evidence that the amino acid sequence of the final protein is determined in the microsomal particle. The evidence for this conclusion is that, although the pH 5 enzyme of guinea-pig liver forms leucine-RNA and isoleucine-RNA at similar rates,[18] these amino acids are incorporated into protein in the proportions in which they exist in hemoglobin.

We wish to express our appreciation to Dr. Henry Borsook, whose encouragement and numerous contributions in this field have greatly aided these studies. It is a pleasure to thank Dr. James Bonner and Dr. Howard Dintzis for many stimulating discussions.

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[1] The term "microsomes" or microsomal particles as used in this paper includes the "microsomes" of tissues such as liver, which consist of ribonucleoprotein particles plus endoplasmic reticulum, as well as "microsomes" from tissues such as reticulocytes, which consist mainly of

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*DELAYED LIGHT EMISSION IN GREEN PLANT MATERIALS: TEMPERATURE-DEPENDENCE AND QUANTUM YIELD**

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Introduction.—The discovery of the delayed light emission of plant materials by Strehler and Arnold in 1951[1] has stimulated a good deal of interest in this rather remarkable property. The emitted light has been shown to be due to an electronic transition between the first excited singlet state of chlorophyll and the ground state.[2, 3] At room temperature, a luminescence is observable from about 0.01 second[4] to several minutes[5] after excitation. Thus the electronic transition cannot be rate-determining, and the process represents neither normal fluorescence nor normal phosphorescence. Indeed, there is some evidence[4, 6] that the decay curve of the luminescence is the resultant of more than one rate-limiting process. Strehler

and co-workers[4, 7] have been able to demonstrate the existence of many relationships between delayed light emission and photosynthesis and thus have been led to interpret the luminescence phenomena as a consequence of the reversibility of some of the enzymatic photosynthetic reactions. However, Tollin and Calvin[6] have shown that the faster-decaying components of the delayed light are present to as low a temperature as $-100°$ C., suggesting that the early processes following light absorption are non-enzymatic in nature. These latter observations, in conjunction with several other types of experimental and theoretical information,[8–15] have suggested an interpretation of the physical processes leading to delayed light emission and, by analogy, to photosynthesis, in terms of semiconductor theory.[16–18]

The earlier investigations in this laboratory[3, 6] have been limited to the study of the light emitted approximately 0.1 second after excitation by a flash discharge. The recent reports of luminescences at still shorter times after excitation[4, 19] have prompted the construction of a device capable of continuously observing the light emission of a sample of plant material from 0.0015 second to about 30 seconds after the onset of flash excitation. The present work describes a series of experiments carried out with this apparatus.

Materials and Methods.—The chloroplast material was prepared as outlined previously.[10] *Chlorella* and *Scenedesmus* were grown in continuous culture in our laboratory, and samples were prepared for the luminescence measurements by centrifugation of a suspension of the algae to obtain a relatively thick paste. In general, measurements were begun within 10 minutes of harvesting.

A block diagram of the apparatus used in the experiments is shown in Figure 1.

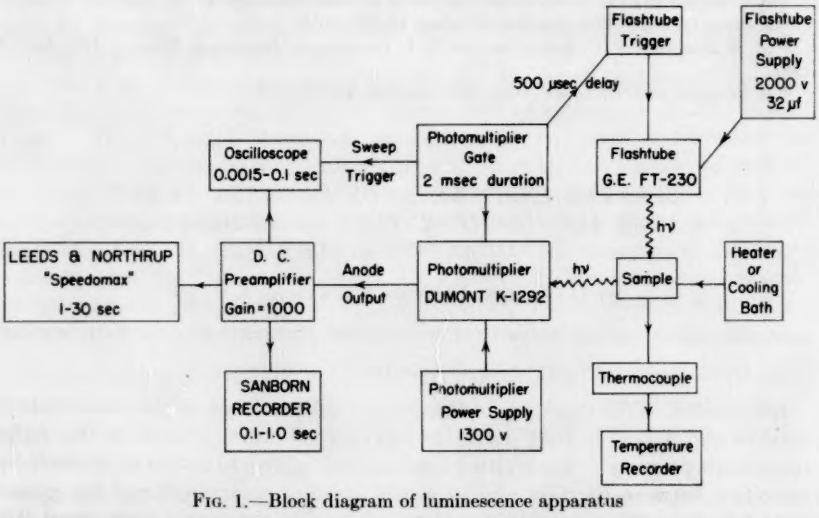


FIG. 1.—Block diagram of luminescence apparatus

Conceptually, it is quite similar to the apparatus described previously,[6] with the exception that the mechanical shuttering system is replaced by an electronic device. This latter involves a "gating" of the photomultiplier (Dumont K-1292) during the exciting light flash through the application of a voltage pulse to the first two

dynodes and the shield, which results in a reversal of their polarity with respect to the photocathode. This serves to prevent most of the photoelectrons liberated at the cathode from being multiplied in the tube. The duration of this gating pulse is continuously variable from a few microseconds to about 5 milliseconds and thus may be matched to the duration of the flash. The gate is also used to start the sweep of a Tetronix 514-D cathode-ray oscilloscope. A delay of about 500 microseconds is introduced between the beginning of the gating process and the "triggering" of the flash, to allow the gate to become fully operative before the light commences. Thus this arrangement permits the onset of the observation of the luminescence to begin in a time limited only by the duration of the flash excitation. The flash tube used in the experiments is the General Electric FT-230, which has the advantage of combining a relatively short duration (about 1.5 milliseconds under the conditions of luminescence measurement) and a small source size (spherical with a diameter of about 4 mm.) with a reasonably high light intensity in a suitable wave-length range. The flash tube is operated at 2,000 volts with a 32-microfarad load capacitor. The triggering system is standard. All electrical leads were kept as short as possible, to achieve optimum pulse-transmission characteristics.

In summary, then, the sequence of events in a measurement is as follows: A push button activates the photomultiplier gate and simultaneously triggers the oscilloscope sweep. Five hundred microseconds later, the flash is fired, and 1.5 milliseconds after this occurs the gate turns off and the photomultiplier is then ready to observe light from the sample.

The exciting light is focused onto the sample through a filter system consisting of a Corning No. 5113, a Corning No. 4303, and a Corning special infrared filter. This combination passes a band of light of wave lengths between 3700 to 4700 A having a maximum at about 4150 A. A Corning No. 2030 and another Corning special infrared filter is placed between the sample and the photomultiplier. This latter filter system allows only those wave lengths between 6500 and about 9500 A to reach the photomultiplier. Such an arrangement of filters minimizes the light from the flash discharge which is incident upon the photomultiplier during excitation, although it by no means completely eliminates the scattering problem. However, no transient phenomena or saturation effects in the photomultiplier response are observed. The geometrical arrangement of exciting source, sample, and photomultiplier is essentially the same as that reported previously.[6]

The photomultiplier anode is supplied with a 2-megohm load resistor, and the voltage developed across this resistance is fed directly into a Sanborn stabilized d.c. preamplifier (gain = 1,000). The preamplifier, in turn, feeds the oscilloscope, a Sanborn Model 151 recorder, and a Leeds and Northrup "Speedomax" recorder. The photomultiplier is normally operated at 1,300 volts.

For the measurement of the quantum yield of the luminescence, the photomultiplier was calibrated using a National Bureau of Standards lamp and a bolometer. The photomultiplier was then used to measure both the light incident on the sample from the flash and the light emitted from the sample. It was assumed that all the light reaching the sample was absorbed. Such an assumption is probably quite satisfactory, inasmuch as the samples used were almost black in appearance. Appropriate corrections were applied for the filters, for the geometry of the system, and for self-absorption in the sample. The values obtained for the

quantum yield of the light emitted between 0.0015 and 30 seconds after excitation are probably accurate only to within a factor of 2 or 3. Furthermore, the yield is too low in terms of total light emitted, inasmuch as we are unable to detect emissions of duration longer than 30 seconds or shorter than 0.0015 second. It is unlikely, however, that inclusion of this energy would raise the quantum yield value by more than an order of magnitude, since extremely sensitive quantum counting devices are needed to observe the longer-term emissions[5] and the present results indicate a steadily decreasing quantum yield as one goes to shorter times after excitation (see below).

Results.—Some typical decay-curve data obtained from *Chlorella* at 21° C. are shown in Figure 2. All the results presented in this section have been obtained from data of this type by an evaluation of the luminescence intensity at various times after excitation. It is apparent that the signal-to-noise ratio is generally quite good, and thus fairly accurate decay curves may be calculated.

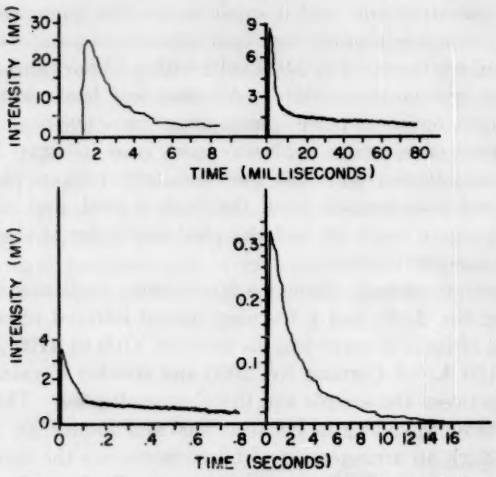


Figure 2. Tracings of decay-curve data obtained from *Chlorella* at 21° C. The three longer-term curves were obtained with a single flash excitation. The fastest curve (0.0015–0.009 second) was obtained independently.

The results of the quantum yield measurements for whole spinach chloroplasts, *Scenedesmus*, and *Chlorella* are shown in Table 1. The total light emission from different samples of the same organism shows some variation in intensity, presumably due to differences in the physiological state of the material. However, this rarely amounts to more than a factor of 2. Strehler and Arnold[1] have reported a figure of 10^{-6} for the ratio of the quanta emitted by *Chlorella* suspensions near the beginning of the observed decay curve to the quanta absorbed. The quantum yields reported in the present work are consistent with this number.

TABLE 1

ABSOLUTE QUANTUM YIELDS OF LUMINESCENCE OF VARIOUS GREEN PLANT MATERIALS

Material	(Quanta Emitted/Quanta Absorbed)
Spinach chloroplasts	4×10^{-7}
Scenedesmus	3×10^{-6}
Chlorella	4×10^{-6}

A comparison of the room-temperature emissions of spinach chloroplasts, *Scenedesmus*, and *Chlorella* is given in Table 2. The choice of time ranges is somewhat

TABLE 2

ROOM-TEMPERATURE LUMINESCENCE OF VARIOUS GREEN PLANT MATERIALS

MATERIAL	INTEGRATED LIGHT INTENSITIES IN VARIOUS TIME RANGES AFTER EXCITATION HAS CEASED (NORMALIZED TO 0.001–0.01-SEC. RANGE OF SPINACH CHLOROPLASTS) 0.001–0.01 Sec.	0.01–0.1 Sec.	0.1–1.0 Sec.	1.0–10.0 Sec.	TOTAL INTEGRATED LIGHT INTENSITY (NORMALIZED TO SPINACH CHLOROPLASTS)
Spinach chloroplasts	1.0	1.5	2.6	4.1	1.0
Chlorella	3.2	6.2	24.1	53.5	9.4
Scenedesmus	2.0	4.1	17.3	36.0	6.4

TABLE 3

TEMPERATURE-DEPENDENCE OF SPINACH CHLOROPLAST LUMINESCENCE

Temp. (° C.)	Time after Excitation Has Ceased (Sec.)	Integrated Light Intensities (Normalized to 0.001–0.01 Range 21° C.)	Per Cent of Total Integrated Intensity	Total Integrated Light Intensity (Normalized to 21° C.)
21	0.001–0.01	1.0	10.8	1.0
	0.01–0.1	1.5	16.3	
	0.1–1.0	2.6	28.3	
	1.0–5.0	4.1	44.6	
11	0.001–0.01	2.3	53.5	0.42
	0.01–0.1	0.84	19.5	
	0.1–1.0	0.66	15.3	
	1.0–5.0	0.50	11.7	
5	0.001–0.01	3.1	40.5	0.80
	0.01–0.1	1.1	14.4	
	0.1–1.0	1.2	15.7	
	1.0–5.0	1.95	25.4	
0	0.001–0.01	3.5	68.4	0.56
	0.01–0.1	1.6	31.3	
	0.1–1.0	0.02	0.3	
	1.0–5.0	0	0	
−4	0.001–0.01	3.8	73.1	0.55
	0.01–0.1	1.4	26.9	
	0.1–1.0	0	0	
	1.0–5.0	0	0	
−18	0.001–0.01	5.9	73.8	0.86
	0.01–0.1	2.1	26.2	
	0.1–1.0	0	0	
	−1.0–5.0	0	0	
−36	0.001–0.01	13.1	53.9	2.6
	0.01–0.1	9.1	37.4	
	0.1–1.0	2.1	8.7	
	1.0–5.0	0	0	
−62	0.001–0.01	7.5	49.3	1.7
	0.01–0.1	5.2	34.2	
	0.1–1.0	2.7	16.5	
	1.0–5.0	0	0	
−95	0.001–0.01	3.7	80.8	0.50
	0.01–0.1	0.78	17.0	
	0.1–1.0	0.10	2.2	
	1.0–5.0	0	0	
−128	0.001–0.01	1.7	77.3	0.24
	0.01–0.1	0.5	22.7	
	0.1–1.0	0	0	
	1.0–5.0	0	0	
−168	0.001–0.01	1.7	77.3	0.24
	0.01–0.1	0.5	22.7	
	0.1–1.0	0	0	
	1.0–5.0	0	0	

arbitrary. It is seen that the algae give a much higher absolute yield of luminescence than do the chloroplasts (see also Table 1) and that this higher yield is mainly due to a disproportionately greater quantity of slowly decaying light obtained from

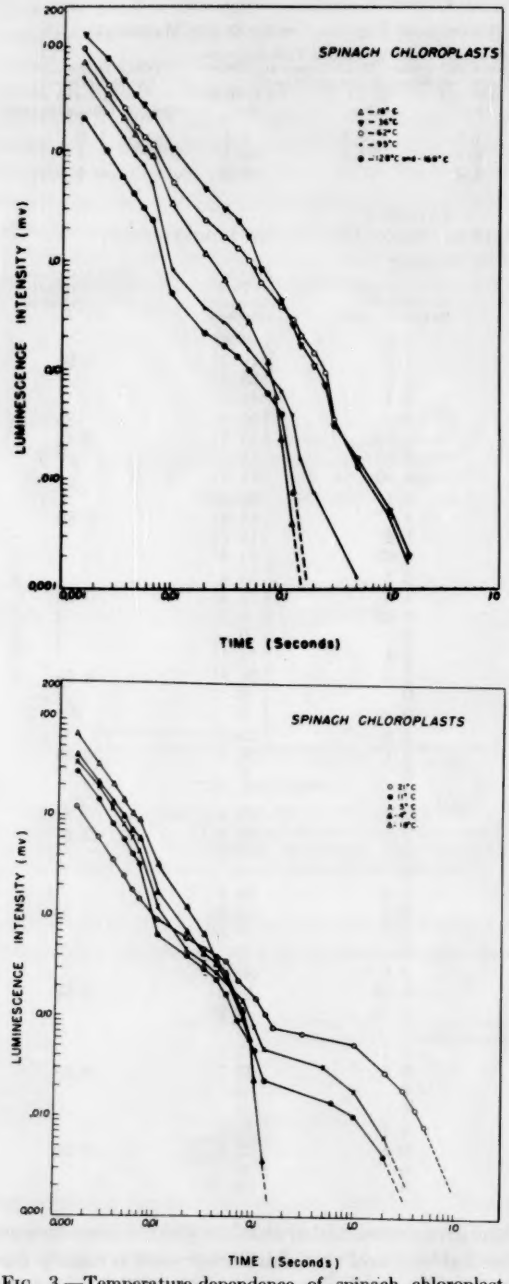


FIG. 3.—Temperature-dependence of spinach chloroplast luminescence.

the former as compared with the latter. The decay curves for *Chlorella* and for *Scenedesmus* are, in fact, quite similar, differing essentially only by a scale factor.

In Figures 3, 4, and 5 and Tables 3 and 4 are given the results of experiments in which the luminescence of spinach chloroplasts and of *Chlorella* is studied as a function of the temperature. It is apparent that the temperature-dependence of the light emission in both types of material is quite complex. Furthermore, at no temperature can the decay curve be represented by a simple kinetic expression (either unimolecular or bimolecular). The most striking change upon cooling in both materials is the fairly rapid changeover from a decay curve in which most of the light is emitted in the longer times (greater than 0.1 second) to a decay curve in which essentially all the emission decays rapidly (see Tables 3 and 4, fourth col.). Another change of interest is the general (although not quite monotonic) increase in the absolute intensity present in the faster components as one cools to intermediate temperatures, followed by a decrease in this intensity as one cools still further (see Fig. 5). This effect is particularly large in spinach chloroplasts and results in

an almost threefold increase in the total integrated intensity obtained at −36° C. as compared with that obtained at 21° C. (see Table 3, fifth col.). In *Chlorella*, it appears that what would otherwise be a peak in total integrated

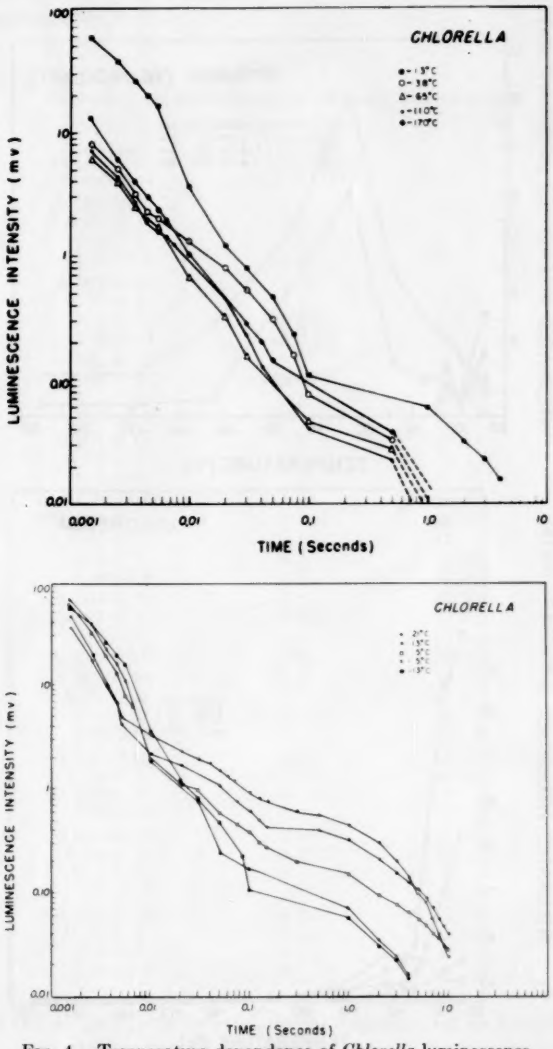


FIG. 4.—Temperature-dependence of *Chlorella* luminescense

intensity at intermediate temperatures is masked by the rapid decrease in the intensity of the slowly decaying light (which represents a much larger percentage of the total intensity in *Chlorella* than it does in chloroplasts). This results in a monotonic decrease in the total integrated intensity, with the suggestion of a

possible maximum appearing as a leveling-off at intermediate temperatures (see Table 4, fifth col.). It is of interest that the maximum of the fastest component comes at a higher temperature in *Chlorella* than it does in chloroplasts.

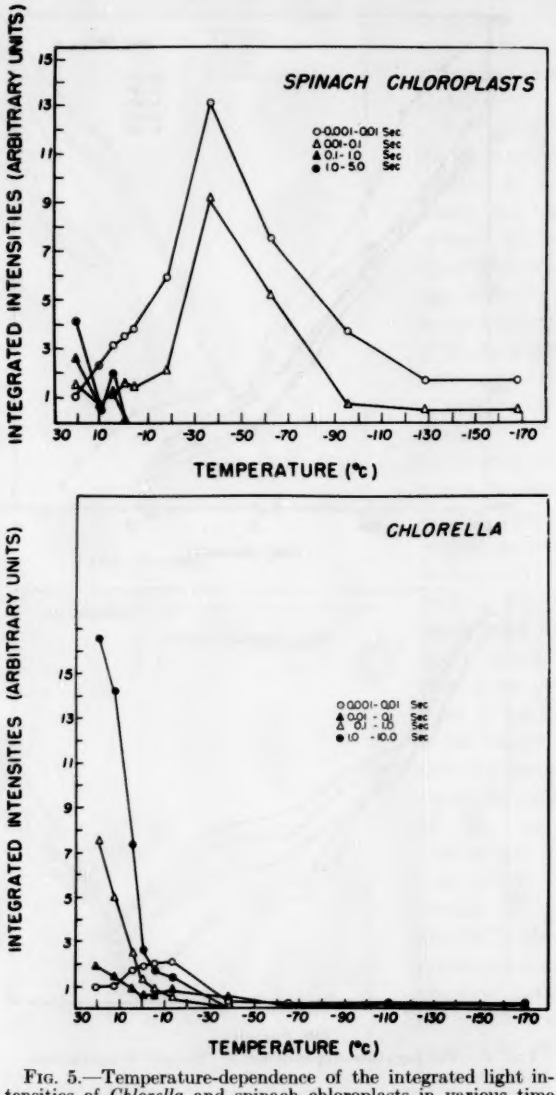


FIG. 5.—Temperature-dependence of the integrated light intensities of *Chlorella* and spinach chloroplasts in various time ranges. The integrated intensity values have been normalized for each organism to the 0.001–0.01-second range at 21° C.

Experiments involving the use of various filters between the sample and the photomultiplier demonstrate that the emission in the first few milliseconds after

excitation has the same wave-length distribution as do the slower components[3] (i.e., emission is occurring from the first excited singlet state of chlorophyll) at both 21° and −170° C. This is a further indication of the failure of the triplet state of chlorophyll to emit,[3] although it may be involved in the sequence of events leading to luminescence.[18]

TABLE 4

TEMPERATURE-DEPENDENCE OF *Chlorella* LUMINESCENCE

Temp. (° C.)	Time after Excitation Has Ceased (Sec.)	Integrated Light Intensities (Normalized to 0.001–0.01 Range 21° C.)	Per Cent of Total Integrated Intensity	Total Integrated Light Intensity (Normalized to 21° C.)
21	0.001–0.01	1.0	3.7	1.0
	0.01–0.1	1.9	7.1	
	0.1–1.0	7.5	27.7	
	1.0–10.0	16.6	61.5	
13	0.001–0.01	1.01	4.6	0.80
	0.01–0.1	1.4	6.3	
	0.1–1.0	5.0	23.0	
	1.0–10.0	14.3	66.1	
5	0.001–0.01	1.7	13.5	0.46
	0.01–0.1	0.9	7.0	
	0.1–1.0	2.5	20.0	
	1.0–10.0	7.4	59.5	
0	0.001–0.01	1.9	28.7	0.24
	0.01–0.1	0.6	9.4	
	0.1–1.0	1.3	19.9	
	1.0–10.0	2.7	42.0	
−5	0.001–0.01	2.0	38.1	0.19
	0.01–0.1	0.65	12.5	
	0.1–1.0	0.9	17.0	
	1.0–10.0	1.7	32.4	
−13	0.001–0.01	2.1	43.3	0.18
	0.01–0.1	0.85	17.3	
	0.1–1.0	0.5	10.3	
	1.0–10.0	1.4	29.1	
−38	0.001–0.01	0.3	40.1	0.03
	0.01–0.1	0.5	59.8	
	0.1–1.0	0	0	
	1.0–10.0	0	0	
−65	0.001–0.01	0.23	65.2	0.013
	0.01–0.1	0.17	34.8	
	0.1–1.0	0	0	
	1.0–10.0	0	0	
−110	0.001–0.01	0.25	59.6	0.015
	0.01–0.1	0.17	40.4	
	0.1–1.0	0	0	
	1.0–10.0	0	0	
−170	0.001–0.01	0.29	55.1	0.020
	0.01–0.1	0.24	44.9	
	0.1–1.0	0	0	
	1.0–10.0	0	0	

In Figure 6 the decay curves for spinach chloroplasts and for *Chlorella* at 21° and at −165° C. are plotted as log intensity versus time. The low-temperature curves for both materials are quite similar in shape. It is possible to interpret these curves as being the resultant of two unimolecular luminescent processes having half-lives of about 1–2 milliseconds and about 30 milliseconds, respectively. The validity of such an interpretation is, of course, open to some question. However, it is of interest that, as one approaches these low temperatures, the changes in the

luminescence for a given temperature increment become progressively smaller, and, in fact, the decay curve for spinach chloroplasts does not undergo any measurable changes between −128 and −168° C. This would suggest a corresponding simplification in mechanism.

Some interesting effects are obtained if one allows the samples to age on the sample holder in the dark at room temperature. A typical result is shown in Table 5. A marked decrease in the total integrated light intensity is observed

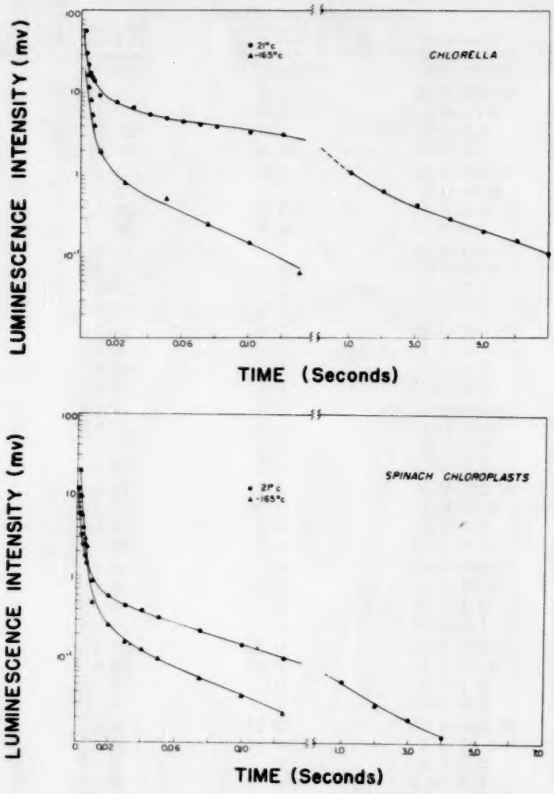


FIG. 6.—Luminescence decay curves of *Chlorella* and spinach chloroplasts at two temperatures.

after 20 hours of aging, with the slower components having decreased to a much greater extent than the faster ones. The material is quite dry and hard at this time. If one rewets the sample with a drop of water, one can achieve some reactivation. However, the percentage of the original intensity present after wetting is much greater for the faster components than for the slower ones. Even after as much as 10 days of aging, the fast decays are still present, and some reactivation of the slow decays is possible.

Discussion.—It is highly unlikely that a temperature-dependence as complex as that observed in the present experiments for delayed light emission can be a reflec-

tion of an underlying mechanistic simplicity. Indeed, the general features of the changes in the luminescence decay curves upon cooling may be readily accounted for qualitatively by postulating a series of either parallel or sequential rate-limiting processes as constituting the basic kinetic pattern of the luminescence phenomenon. Such a complicated sequence of events leading to an electronically excited chlorophyll molecule is consistent with what is known about the photosynthetic pathway and about metabolic processes in general.

In terms of the above picture, one can explain the increase in the intensity of the faster light emissions at intermediate temperatures as being due either to the undetected presence of still faster decay processes of high yield whose rates are decreased upon cooling or to a "freezing-out" of the slower mechanisms, with a corresponding increase in the amount of energy being emitted in the faster processes. It is not possible to say which of these points of view is applicable to the present situation. The rationale for the subsequent decrease in intensity upon further cooling is obvious.

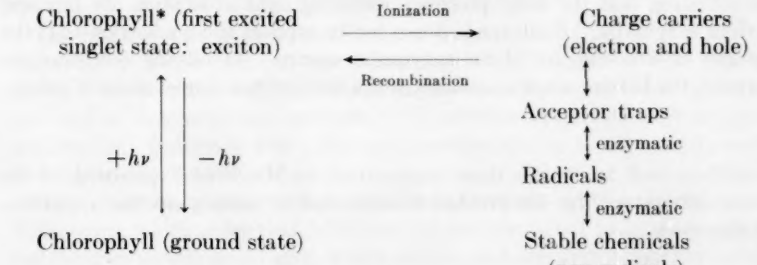
The most significant result of the present study is the observation of a substantial luminescence decay at temperatures as low as $-170°$ C. This strongly suggests that the basic mechanisms of the early processes following light absorption do not

TABLE 5

EFFECT OF AGING AND REWETTING ON ROOM-TEMPERATURE *Scenedesmus* LUMINESCENCE

	INTEGRATED LIGHT INTENSITIES IN VARIOUS TIME RANGES AFTER EXCITATION HAS CEASED (NORMALIZED TO 0.001–0.01-SEC. RANGE OF FRESH MATERIAL)				TOTAL INTEGRATED LIGHT INTENSITY (NORMALIZED TO FRESH MATERIAL)
	0.001–0.01 Sec	0.01–0.1 Sec	0.1–1.0 Sec	1.0–10.0 Sec	
Fresh	1.0	2.05	8.7	18.0	1.0
Aged 20 hours	0.20	0.23	0.41	0.12	0.03
Rewet after aging	0.63	0.95	2.3	2.25	0.21
Per cent of original intensity recovered	63	46	26	12	21

involve the migration of atomic nuclei and thus are purely physical. This is, of course, quite consistent with the general features of the semiconductor mechanism proposed earlier.[16–18] According to this hypothesis, the pathway of the light quantum may be depicted in terms of the following scheme (for simplicity, the triplet state of chlorophyll has not been included in the sequence):



If one granted the validity of separating the $-170°$ C. decay into two components, one could identify the faster of these decays with charge-carrier lifetime and the slower decay with the emptying of a shallow trapping level. Both these processes

should be relatively temperature-independent over the range studied. It is striking that the time constant of the slow decay (~0.03 second) is of the same order of magnitude as those observed for (*a*) the minimum dark time for photosynthesis of *Chlorella* in flashing light by Emerson and Arnold;[20] (*b*) the corresponding dark time for the Hill reaction in *Chlorella* by Clendenning and Ehrmantraut;[21] (*c*) the decay time in *Chlorella* for absorption spectra changes at 5150 A by Witt;[22] and (*d*) the minimum dark time for oxygen production of the Hill reaction in *Scenedesmus* in the presence of thioctic acid by Bradley and Calvin.[17] Such a correlation would argue that the rate-limiting step in these processes is physical rather than enzymatic.

The fact that algae yield a much greater light intensity in the slow decays than do chloroplasts is probably a consequence of the partial removal of enzymes and smaller molecules in the preparation procedure. This suggests chemical transformations as the rate-limiting steps of the slower components. An explanation in these terms would also be consistent with the large temperature coefficient of these components as observed in the cooling experiments (Tables 3 and 4), with the aging experiments (Table 5), and with the results of Strehler and co-workers.[4, 7]

The extremely low quantum yields observed in the present experiments are in accordance with the interpretation of delayed light emission as an indication of the reversibility of at least part of the photosynthetic pathway. These low values are probably a reflection of the high efficiency with which the absorbed quanta pass over into the chemical processes involved in photosynthesis. It is interesting to note that the much higher quantum yield of fluorescence[13] ($\sim 10^{-2}$) suggests a considerably lower order of efficiency of quantum conversion for the earliest physical stages.

Summary.—A device has been constructed which is capable of recording the decay curve of green-plant luminescence from 0.0015 second to approximately 30 seconds after excitation by a flash discharge. Absolute quantum yield measurements of the emitted light give values of the order of 10^{-6} for *Chlorella* and *Scenedesmus* and 10^{-7} for spinach chloroplasts. Such low yields are in accord with the interpretation of delayed light emission as a reversal of photosynthesis. The luminescence has been found to exhibit an extremely complex temperature-dependence, which is suggestive of a multiprocess mechanism. A substantial luminescence decay is measurable at temperatures as low as $-170°$ C. This is interpreted as demonstrating that the early processes following light absorption are physical rather than enzymatic. Evidence is presented to support the contention that the later stages of emission are of an enzymatic nature. At all the temperatures investigated, the luminescence originates in the first excited singlet state of chlorophyll.

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*THE BIOSYNTHESIS OF ISOLEUCINE AND VALINE IN NEUROSPORA CRASSA**

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INTRODUCTION

The possible role of α-aceto-β-hydroxy-butyric acid (AHB) as a precursor of isoleucine in *Escherichia coli* was pointed out by Umbarger.[1] The related compound, α-acetolactic acid (AL) has been shown to be a precursor of valine in *E. coli*[2] and in *Saccharomyces cerevisiae*.[3] In addition, a mutant of *Neurospora* that requires both isoleucine and valine for growth is known to accumulate both AHB and AL in the medium.[4]

This communication describes briefly experiments with cell-free extracts of *Neurospora*, which show that AHB and AL are converted to isoleucine and valine via the previously[5] postulated intermediates, α,β-dihydroxy-β-methylvaleric acid (DHI) and α,β-dihydroxyisovaleric acid (DHV), respectively. Evidence is also given that this conversion from the β-keto acids, AHB and AL, to the dihydroxy acids is a two-step process, probably involving the intermediate formation of α-keto-β-hydroxy acids, as indicated in Figure 1.

MATERIALS

Three strains of *N. crassa* were used. Strain 16117 requires isoleucine and valine for growth and accumulates the dihydroxy acids, DHI and DHV, in its growth medium.[6] Strain 304 also requires isoleucine and valine for growth but is blocked at a different step in metabolism than 16117, since it accumulates AHB and AL rather than the dihydroxy acids.[4] Emerson 5256 is the wild-type standard strain which grows on an unsupplemented minimal medium. The minimal medium described by Vogel,[7] supplemented for the mutant strains with isoleucine and valine, was used to grow all cultures.

DL-α-Acetolactic acid and DL-α-aceto-α-hydroxybutyric acid were synthesized by the method described by Krampitz.[8] In the synthesis of AHB, ethyl iodide

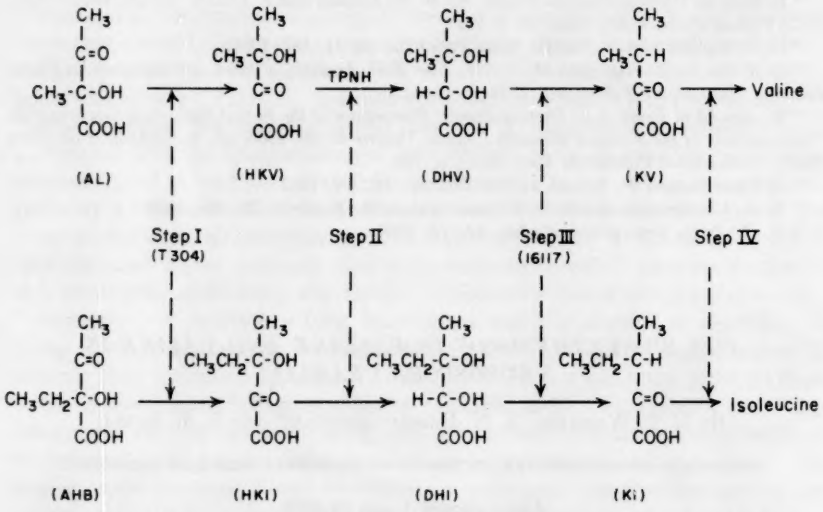

FIG. 1.—Proposed sequential steps in the formation of valine and isoleucine from α-acetolactate and α-aceto-α-hydroxybutyrate in *Neurospora*. The entire sequence of reactions is carried out by cell-free extracts of the wild type; strains 304 and 16117 appear to be blocked at the indicated steps.

was used in place of methyl iodide employed in the synthesis of AL. The keto-hydroxy acids, α-keto-β-hydroxy-β-methylvaleric acid (HKI) and α-keto-β-hydroxyisovaleric acid (HKV), were synthesized by the method of Sprinson and Chargaff[9] from the respective α-keto acids prepared according to Meister.[10] HKV and HKI prepared as above contained small amounts of α-hydroxy-isobutyric acid and α-hydroxy-α-methyl butyric acid, respectively. The HKI obtained was optically inactive, although the keto acid used as a starting material was optically active.

Glucose-6-phosphate (G-6-P) and triphosphopyridinenucleotide (TPN) were obtained from Nutritional Biochemicals Corporation. Reduced TPN (TPNH) and diphosphopyridinenucleotide (DPN) were obtained from Sigma Chemical Co.

RESULTS

Enzymatic Conversion of AHB to DHI.—*Neurospora crassa* 16117 was cultured for 48 hours at 37° with constant shaking in minimal medium supplemented with 4 μmoles of DL-valine and 1.5 μmoles of DL-isoleucine per liter. The mycelium was filtered, washed with distilled water, and then ground to a fine powder under liquid nitrogen in a mortar. The powder was then homogenized with two to three times its weight of distilled water in a Ten Broeck apparatus. The resulting slurry was centrifuged in the cold (0–4°) at 18,000 × *g*.

The cell-free supernatant fluid thus obtained was tested for its activity in converting AHB to DHI as follows: Each assay tube contained AHB (200 μM), TPN (1 μM), G-6-P (400 μM), enzyme extract (1.0 ml.; protein, 20 mg/ml), and sufficient 0.1 *M* Tris buffer at pH 8.0 to bring the total volume to 4.9 ml. Suitable controls were always included. The reaction mixtures were incubated for 7 hours at 30°. The mixtures were then acidified with hydrochloric acid to a pH of 1.5 to 2.0 and heated at 100° for 5 minutes (or left at room temperature overnight) to decarboxylate unreacted AHB. The mixtures were extracted several times with ethyl ether, and the combined ether extracts were dried with anhydrous Na_2SO_4 and the ether evaporated *in vacuo*. The residue was dissolved in a small

TABLE 1

CHROMATOGRAPHIC PROPERTIES OF COMPOUNDS OF INTEREST IN BIOSYNTHESIS OF ISOLEUCINE AND VALINE

COMPOUND	R_f IN SOLVENT (1) Butanol, Acetic Acid, Water	R_f IN SOLVENT (2) Benzene, Ether, Formic Acid	PERIODATE REACTION OF SPOT
α,β-Dihydroxy-β-methylvaleric acid (DHI)	0.80	0.53	Yellow
α,β-Dihydroxyisovaleric acid (DHV)	0.61	0.33	Yellow
α-Hydroxybutyric acid	0.82	0.70	No reaction
Lactic acid	0.66	0.52	No reaction
α-Keto-β-hydroxy-β-methylvaleric acid (HKI)	0.54	0.81	White
α-Keto-β-hydroxy-isovaleric acid (HKV)	0.25	0.32	White

quantity of ether and spotted on Whatman No. 1 paper, along with reference standards of DHI, DHV, α-hydroxybutyric acid, and lactic acid. Ascending chromatography was carried out, using two different solvent systems: (1) *n*-butanol–acetic acid–water (4:1:1 *v/v*) and (2) ether-benzene–formic acid–water[6] (70:30:12:10 *v/v*; upper phase). After air-drying at room temperature, acidic compounds were detected by spraying with a 0.04 per cent alcoholic solution of bromcresol green (pH 7.0). The yellow acid spots were located and the papers sprayed on the reverse side with a 0.5 per cent solution of sodium metaperiodate. After a 5-minute interval they were sprayed with a benzidine reagent.[11] Glycols and other compounds cleaved by periodate give white or lightly colored spots on a blue to purple background after this treatment. Table 1 gives the R_f values and the periodate reactions for the compounds of interest in this study in the two solvent systems.

The reaction mixtures which initially contained AHB, crude enzyme extract, TPN, and G-6-P gave significant quantities of a compound whose R_f and periodate reaction were identical with those of authentic DHI. Traces of DHI were noted when TPN, but not G-6-P, was present. No significant amounts of DHI were formed with TPN, G-6-P, and enzyme, enzyme alone, or with substrate or enzyme omitted.[12]

Confirmation of the identity of the substance as DHI was obtained after an adequate sample was synthesized by incubating 1.5 m moles of AHB with appropriate amounts of TPN, G-6-P, and enzyme at pH 8.5 for 6.5 hours at 30°. The ether-soluble acids were separated by chromatography on several sheets of Whatman No. 3 MM paper, and the DHI zone, located by spraying guide strips with bromocresol green and periodate, was eluted with water. The eluate of DHI was neutralized with dilute NaOH and lyophilized. The dry residue (37 mg.) corresponded to 218 μ moles of the sodium salt of DHI. If only one isomer of DL-α-aceto-α-hydroxybutyrate is active in this system, then approximately 29 per cent of the available initial substrate was converted to DHI. The infrared spectrum of the isolated sodium salt (1 per cent in KBr) was identical with that of an authentic sample of DHI over the range 2–16 μ.

Conversion of DHI to Isoleucine.—Further proof of the identity of the enzymatically synthesized compound was obtained by converting the purified salt described above to isoleucine by enzyme extracts of wild-type *Neurospora*, Em 5256. The reaction mixtures contained the following: 1.0 ml. of the freshly prepared cell-free extract (*ca.* 5 mg. proteins per ml.), 10 μg. pyridoxal phosphate, 1 μmole of $FeSO_4$, 50 μmoles of L-valine or L-phenylalanine, 2.4 mg. of the isolated DHI (or 2.4 mg. of the standard DHI), and 1.0 ml. of 0.1 *M* Tris buffer, at pH 8.0. The enzyme extract contained L-cysteine (3.3×10^{-3} *M*) added during preparation.[13] The mixtures were incubated for 5 hours at 37°, and the protein precipitated by heating after the addition of 1.0 ml. of 3 *M* sodium acetate buffer, pH 4.5. Chromatographic analysis of the protein-free mixtures showed the presence of isoleucine; the control mixtures showed no detectable amounts of isoleucine. The amount of isoleucine synthesized was determined by bioassay with *Leuconostoc mesenteroides* P-60.[14] The results are presented in Table 2.

TABLE 2*

COMPARATIVE CONVERSIONS OF SYNTHETIC AND ENZYMATICALLY SYNTHESIZED DHI TO ISOLEUCINE BY CELL-FREE EXTRACT OF WILD-TYPE *Neurospora*

Substrates	Isoleucine Formed (μmoles)
Isolated DHI + L-valine	8.4
Isolated DHI + L-phenylalanine	2.5
Standard DHI + L-valine	9.1
Standard DHI + L-phenylalanine	3.3

* See text for conditions. 14.1 μmoles of DHI were present initially in all cases.

Conversion of AL to Valine via DHV.—By procedures altogether similar to those described above, it has been possible to demonstrate the conversion of AL to DHV by cell-free extracts of strain 16117. DHV was isolated in a yield of 7.4 per cent of the original AL by incubating the following mixture for 6 hours: 20.0 ml. of enzyme extract (25 mg. protein per ml.) 1 m mole of AL, 10 mg. TPN, and 1.8 m moles of G-6-P, and 20 ml. of 0.1 *M* Tris buffer at pH 8.5. The isolated DHV was identical with the standard sample in its chromatographic behavior and infrared spectrum. Its identity was further confirmed by converting it enzymatically to valine, as described in the case of DHI, with a cell-free extract of wild-type *Neurospora*.

Enzymatic Synthesis of DHI and DHV from HKI and HKV.—The transformation of the β-keto-α-hydroxy acids, AHB and AL, to the dihydroxy acids, DHI and DHV, requires a rearrangement and a reduction. If rearrangement occurs first, as shown in Figure 1, Step *I*, then α-keto-β-hydroxy-β-methylvaleric acid (HKI) and α-keto-β-hydroxy-isovaleric acid (HKV) would be intermediates in the formation of DHI and DHV, respectively. If this were true, HKI and HKV should be reduced by the enzyme extract and TPNH to yield DHI and DHV, respectively.

Cell-free extracts of the three strains of *Neurospora* were prepared as previously described. The enzyme activity was measured by following the change in optical density at 340 mμ of a reaction mixture consisting of dialysed enzyme extract, TPNH (0.1 μmole), HKI or HKV (10–20 μmoles), and 0.1 *M* Tris buffer (pH 7.5) in a total volume of 1.0 ml. By definition, a unit of ketohydroxy acid reductase activity was contained in that amount of extract which would cause a change in O.D. of 0.1 in 5 minutes, under the conditions employed. Table 3 gives the enzyme activities of crude and purified extract of the three strains.

Proof for the conversion of the α-keto-β-hydroxy acids to the dihydroxy acids was obtained by actual isolation of the latter as follows: 800 μmoles of HKI and 400 μmoles of HKV were separately incubated for 5.5 hours at 37° with 1 m mole of G-6-P, 5 μmoles of TPN, 15 mg. of crude lyophilized extract of 16117, and sufficient 0.1 *M* Tris buffer at pH 7.5 to bring the total volume to 15 ml. The DHI and DHV formed in the reaction were isolated by chromatography on thick paper, and the sodium salts prepared. 166 μmoles of DHI (41 per cent conversion on the assumption that only one isomer is involved) and 154 μmoles of DHV (39 per cent conversion) were isolated. The compounds were identical in chromatographic properties and infrared spectrum with standard DHI and DHV. They were also converted to isoleucine and valine, respectively, by extract of wild-type *Neurospora*.

Specificity and Stability of the α-Keto-β-Hydroxy Acid Reductase.—The enzyme that catalyzes the reduction of the α-keto-β-hydroxy acids is present in all three strains of *Neurospora*. It has been purified ten to fifteen fold by fractional precipitation with $AlCl_3$ and $(NH_4)_2SO_4$ (Table 3). The purified enzyme preparations

TABLE 3*

COMPARATIVE ACTIVITIES OF CRUDE AND PARTIALLY PURIFIED KETOHYDROXYACID REDUCTASE TOWARD TWO SUBSTRATES

ENZYME FROM *N. crassa*, STRAIN	SUBSTRATE: HKI		HKV	
	Crude Extract	Purified	Crude Extract	Purified
Em 5256	2.5	16	2.3	20
16117	3.8	38	4.8	50
304	1.5	32	2.0	36

* Figures represent specific activities (units of activity per mg. of protein) of the crude versus the partially purified enzyme. See text for conditions.

showed no G-6-P dehydrogenase activity in the presence of TPN and showed no appreciable oxidation of TPNH. Pyruvic, α-keto-β-methylvaleric, and α-keto-isovaleric acids were not reduced in the presence of TPNH. The enzyme is specific for TPNH, DPNH being entirely inactive in the reaction with the ketohydroxy acids.

Apparent dissociation constants for HKI and HKV were determined in the presence of excess TPNH and the partially purified enzyme preparation from

16117. The K_m for HKI was approximately 3.8×10^{-4} mole per liter. The corresponding value for HKV was 1.6×10^{-3}.

Crude or purified preparations of the enzyme are not inactivated by dialysis or by lyophilization. Such dried preparations have not lost activity upon storage for periods up to 3 months at $-20°$ C.

DISCUSSION

The results presented above clearly demonstrate that AHB and AL can be converted to isoleucine and valine, respectively, by crude, cell-free extracts of *N. crassa*. The conversion involves the intermediate formation of DHI and DHV previously postulated to be intermediates in the synthesis of these amino acids.[5] The biosynthesis of DHI and DHV from AHB and AL requires a rearrangement of the β-keto-α-hydroxy acids, as suggested by Adelberg[15] and Strassman *et al.*[16] The subsequent postulate[17] that γ-hydroxy-β,γ-dimethyl-α-ketoglutaric acid and γ-hydroxy-γ-methyl-α-ketoglutaric acid (HMKG) rather than AHB and AL, might be intermediates in formation of isoleucine and valine is not tenable in view of the results obtained herein.[18]

The possibility that an α-keto-β-hydroxy acid might serve as an intermediate prior to the reduction step was pointed out, without experimental support, by Meister.[19] The demonstration herein of an enzyme in *Neurospora* that specifically reduces α-keto-β-hydroxy acids to the dihydroxy acids in the presence of TPNH provides experimental support for this suggestion and indicates that rearrangement of the carbon chain precedes reduction, although the converse possibility is not conclusively eliminated by the data.

We have been unable to detect any accumulation of HKI and HKV in the conversion of AHB and AL to DHI and DHV, even when the supply of TPNH is eliminated. However, we have noticed in the medium of 16117, which is known to be blocked in the conversion of DHI and DHV to the corresponding α-keto acids, a compound whose chromatographic properties are similar to HKI. Isolation of this compound in sufficient quantity is necessary for further characterization. The existence of these intermediates is also indicated by an increase in the rate of TPNH oxidation after AHB or AL is pre-incubated with crude extracts, over and above the rate of reduction of AHB or AL alone. The purified enzyme preparation from 16117 also catalyzed formation of DHI and DHV from AHB and AL, respectively, provided that it was supplemented with boiled crude extract. This is in contrast to the corresponding enzyme preparation from strain 304, which contains an active α-keto-β-hydroxy acid reductase, but failed to convert AHB and AL to the dihydroxy acids. Since strain 304 is known to accumulate AHB and AL in its growth medium, it is perhaps blocked at the isomerization step. This would mean that step *I* and step *II* (Fig. 1) are carried out by two different enzymes, and work is in progress on further resolution of the enzymes involved. It is considered likely that the α-keto-β-hydroxy acids are not normally released into the environment and may remain enzyme-bound.

SUMMARY

These experiments have shown that α-aceto-α-hydroxy butyric acid and α-acetolactic acid are converted to α,β-dihydroxy-β-methylvaleric acid and α,β-dihydroxyisovaleric acid by cell-free extracts of *N. crassa*, strain 16117. The

latter compounds, previously postulated to be intermediates in the biosynthesis of isoleucine and valine, are converted to these amino acids by cell-free extracts of *N. crassa*, wild type. An enzyme that actively converts α-keto-β-hydroxy-β-methyl valeric acid and α-keto-β-hydroxyisovaleric acid to the corresponding dihydroxy compounds is present in these extracts. This enzyme specifically requires reduced triphosphopyridine nucleotide and is without activity on closely related substrates such as α-ketoisovaleric acid. All the available evidence indicates that these α-keto-β-hydroxy acids are intermediates in the conversion of β-keto-α-hydroxyacids to the dihydroxy acid precursors of isoleucine and valine.

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[4] R. P. Wagner, A. Bergquist, and H. S. Forrest, unpublished material.

[5] E. A. Adelberg, in W. B. McElroy and B. Glass, *A Symposium on Amino Acid Metabolism* (Baltimore, 1955).

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[9] E. B. Sprinson and E. Chargaff, *J. Biol. Chem.*, **164**, 417, 1946.

[10] A. Meister, *J. Biol. Chem.*, **197**, 308, 1952.

[11] Benzidine 0.5 gm., glacial acetic acid 20.0 ml., ethyl alcohol 80.0 ml.; prepared once each week.

[12] AHB and AL decarboxylate spontaneously under the acid conditions employed. The products acetylethylcarbinol and acetylmethylcarbinol are volatile and are not detected on the chromatograms. However, in addition to the enzymatically formed DHI, a second prominent acidic zone appears on chromatograms of all reaction mixtures containing AHB with or without added enzyme. This compound was identified as α-hydroxybutyric acid apparently formed by "acid-type" cleavage of AHB. Lactic acid is obtained from AL under the same conditions. Lactic acid has been recently obtained from AL by alkali treatment (Böhme and Schneider, *Chem. Ber.*, **91**, 988, 1958). Acetic acid, the other cleavage product, is volatile and is not detected on the chromatograms. We have established that about 10 per cent of the AHB and AL are cleaved in this fashion under the experimental conditions.

[13] Ferrous ion and cysteine were present in the reaction mixtures (J. Myers, Ph.D. thesis, University of California, 1956).

[14] L. M. Henderson and E. E. Snell, *J. Biol. Chem.*, **172**, 15, 1948.

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[18] Dr. E. A. Adelberg has confirmed the conversion of AL to DHV by using crude extracts of *N. crassa* 16117 but was unable to show any conversion of HMKG to DHV by this strain under any conditions tried. He states: "The hypothesis that HMKG and γ-hydroxy-β,γ-dimethyl-α-ketoglutaric acid were intermediates in the biosynthesis of valine and isoleucine was based on the accumulation of the oxidative decarboxylation products of these compounds in the medium of strain 7110. In view of the positive results obtained with AL and AHB reported here, the most likely explanation of these accumulations is that 7110 is blocked prior to formation of AHB and AL and carried out an abnormal condensation, perhaps as an induced enzyme reaction" (personal communication).

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THE PRESENCE OF A_2 ERYTHROCYTES IN A_1 BLOOD

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Small proportions of O or B erythrocytes have been found in the blood of normal A or AB individuals, respectively, by means of the isotope dilution method[1] and direct isolation.[2, 3] This form of blood group admixture—automosaicism—is distinguishable from true chimerism in that the absence of one agglutinogen is independent of unrelated agglutinogens, whereas in the chimera it is accompanied by unrelated differences signifying the foreign origin of a portion of the hematopoietic tissue. The origin of automosaicism is unsolved; it may represent merely a non-uniform development of the cell phenotype, or it may reflect an underlying genetic heterogeneity of the erythropoietic system. Somatic segregation, suggested by Goudie[3] as a basis for such heterogeneity, would lead to homozygosis for the opposing alleles. The exceptional non-A cells in AB individuals would therefore be phenotypically B, and those in heterozygous A individuals would be phenotypically O. It is of interest in this connection to learn that A_1 and A_1B individuals possess exceptional cells having the phenotype of subgroup A_2 or A_2B.

Suitable reagents for recognition of the subgroup in isotope dilution experiments are provided by the seed extracts of *Dolichos biflorus*,[4] specific for A_1, and *Phaseolus limensis*, which agglutinates both A_1 and A_2 cells.[5] The method, described in detail elsewhere,[1] requires a series of agglutinations in which the first batch of cells added to the reaction mixture is labeled with Cr^{51} and all the succeeding additions are unlabeled. At each stage the cells are agglutinated in a pan, transferred to a separatory funnel for a brief settling time, and then the main agglutinated mass is withdrawn, leaving a supernatant that contains free cells and small clumps. The supernatant is sampled for determination of Cr^{51} activity and returned to a pan for the next stage, which begins with the addition of further cells and agglutinin. The labeled agglutinable cells are finally exhausted, and the activity of successive supernatants becomes constant. The samples are centrifuged and washed so that only the Cr^{51} bound to cells is counted. Each sample is corrected for the cumulative dilution of the system, and the ratio of the corrected final activity to that of the initial reaction mixture is the proportion of

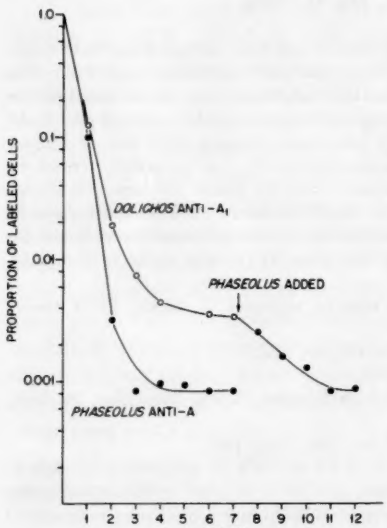

FIG. 1.—Isotope dilution curves with *Phaseolus* and *Dolichos* lectins on cells of A_1 donor S. W. In the experiment begun with *Dolichos* anti-A_1, *Phaseolus* anti-A was added at each stage following the seventh.

inagglutinable cells. In these experiments, the *Dolichos* and *Phaseolus* agglutinins were adjusted to the same titer, 1:128, with A_1B cells. The initial reaction mixtures contained 5 ml. of washed labeled cells, 20 ml. of saline, and 10 ml. of agglutinin. Their activities ranged from 1.8 to 3.5 $\times$ 10^6 counts/minute/ml. The stage additions were 3 ml. of agglutinin and 3 ml. of a 66 per cent suspension of A_1 or A_1B carrier cells. Agglutinations were carried out at 4° C. A well-type scintillation counter was used, with a background of 250 counts/minute.

In Figure 1 the corrected sample activities are plotted against the stage in two experiments with the same A_1 donor. The proportion of phenotypically O cells is just under one per thousand in the experiment with *Phaseolus* anti-A. With

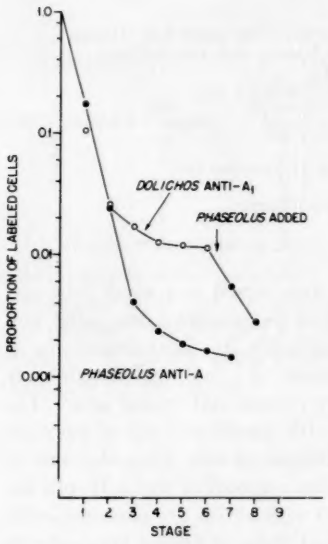

FIG. 2.—Experiments similar to those in Fig. 1 on cells of A_1B donor K. A.

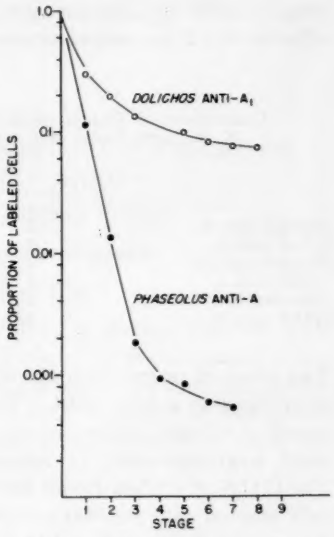

FIG. 3.—Isotope dilution curves on cells of A_1B donor M. E. G. It is noteworthy that the *Dolichos* and *Phaseolus* lectins had the same titer against M. E. G. cells in the standard test, despite the large difference in the proportion of cells inagglutinable by these reagents.

Dolichos anti-A_1, the sum of O and A_2 cells is about 3.5 per thousand. Substitution of anti-A for anti-A_1 in the stages after the seventh produced a decline in the level of the O cells. Figure 2 shows a similar pair of experiments on an A_1B blood, indicating just above one per thousand B cells and 1 per cent A_2B cells. Again the addition of *Phaseolus* lectin after equilibration to *Dolichos* resulted in a further decline, but the experiment was terminated when the volume of the system became limiting before a second equilibrium was reached. Another A_1B blood is analyzed in Figure 3 and contains about one B cell per two thousand and 7 per cent A_2B cells.

Cells inagglutinable by anti-A_1 were obtained from A_1B blood by direct isolation, for comparison with known A_2B cells in ordinary typing procedures. The tech-

nique of isolation described in detail elsewhere[2] is designed to minimize entrapment of inagglutinable cells in the pellets formed by centrifugation in the presence of agglutinin. Washed cells are first agglutinated in a large pan and a preliminary separation accomplished by gravity in a separatory funnel. The supernatant, which still contains mainly agglutinable cells, is then concentrated by centrifugation over high-density fluorocarbon oil. Cells are deposited in a thin layer at the interface, and the layer is subsequently broken into free cells and flakes that are easily separable. The process is repeated on the free cells until no further agglutination is detectable. Significant losses of inagglutinable cells are incurred, but the preparations are fairly pure.

One hundred and fifty milliliters of cells from donor K. A. yielded 0.4 ml. of cells inagglutinable by *Dolichos* anti-A_1. These were thoroughly washed in saline, adjusted to a 2 per cent suspension, and tested with the reagents shown in Table 1.

TABLE 1

COMPARISON OF REACTIONS OF EXCEPTIONAL CELLS SELECTED FROM A_1B (DONOR K. A.) WITH THOSE OF UNSELECTED CELLS AND KNOWN A_2B AND B CELLS

		Selected			
	Unselected Cells	With *Dolichos* Anti-A_1	With *Phaseolus* Anti-A	A_2B (Donor L. R.)	B (Donor C. M.)
Dolichos anti-A_1	4	0	0	0	0
C.D.S. anti-A_1	3	0	0	0	0
Phaseolus anti-A	4	2–3	0	1–2	0
C.D.S. anti-A	4	1–2	±	2	0
Ulex anti-H	±	2	3	2	2–3
C.D.S. anti-B	4	4	4	4	4

Two drops of reagent and one of cell suspension were mixed in a small tube and centrifuged to a firm pellet. The tube was tapped to resuspend the pellet and scored as follows: pellet unbroken, 4; several fragments, 3; many fragments, 2; small fragments only, 1; microscopic agglutination, +; no agglutination, 0. The C.D.S. (Certified Donor Service) reagents are commercial typing sera. The cells selected with *Dolichos* anti-A_1 are compared with unselected cells of the same donor, with known A_2B and B cells, and with cells selected with *Phaseolus* anti-A. By these criteria, the cell suspension remaining after removal of the A_1B cells behaves phenotypically as A_2B, whereas cells selected with anti-A are phenotypically B. The resemblance between the selected cells and those of known type extends even to the cross-reaction with anti-H. In a similar experiment with A_1B donor M.E.G., 170 ml. of cells yielded 6 ml. inagglutinable by *Dolichos* anti-A_1. The ease of isolation in this case was entirely consistent with the result of the isotope dilution experiment on the same donor. The selected M.E.G. cells were used to absorb C.D.S. anti-A and produced a reagent indistinguishable from C.D.S. anti-A_1. Parallel absorptions with unselected M.E.G. cells exhausted C.D.S. anti-A, as expected. In summation, the individuals tested not only possess some cells lacking in A agglutinogen but a larger number in which A_1 is lacking and A_2 present.

Clearly, the exceptional cells of subgroup A_2B cannot be produced by somatic crossing over or by other processes that lead to homozygosis. Even the separation of an ostensible A_1A_2 cistron cannot be invoked, since neither A_1 nor A_2 is ever found in coupling with B. The proportion of cells having A_2 alone seems to be quite uncorrelated with that of cells in which A is absent, suggesting that the proc-

esses giving rise to the two classes are independent. Should this be substantiated, it may narrow the range of speculation as to the origin of the exception cells. At present it is possible that they represent somatic mutation in the proliferating marrow and equally possible that they are phenocopies or, as Koller[6] has expressed it, "serological freaks."

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*GENERATING FUNCTIONS AND THE SEMIGROUP THEORY OF BRANCHING MARKOV PROCESSES**

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1. In this note we consider an approach to the study of Markov processes with a denumerable state space which is based on the properties of the semigroup of operators associated with the generating function of the probabilities defining a particular process. We can define a *process* as a collection $\mathfrak{M} = \{p_{ij}(t)\}$ of real-valued functions on $(0, \infty)$, where i and j range over a fixed denumerable set $\mathfrak{X}$, satisfying the following conditions:

$$\begin{aligned}
&\text{(i)} \quad p_{ij}(t) \geq 0, \qquad t \geq 0;\\
&\text{(ii)} \quad \sum_{k\in\mathfrak{X}} p_{ik}(t) \leq 1, \qquad t \geq 0;\\
&\text{(iii)} \quad p_{ij}(s+t) = \sum_{k\in\mathfrak{X}} p_{ik}(s)p_{kj}(t), \; s, \qquad t \geq 0;\\
&\text{(iv)} \quad p_{ij}(0) = \lim_{t\downarrow 0} p_{ij}(t) = \delta_{ij}.
\end{aligned}$$

Condition (ii) characterizes *dishonest* (or *quasi*) *processes.* When (ii) is strengthened to

$$\text{(ii}') \qquad \sum_{k\in\mathfrak{X}} p_{ik}(t) = 1, \qquad t \geq 0,$$

$\mathfrak{M}$ is called an *honest process;* and in this case the functions $p_{ij}(t)$ are the transition probabilities for a time-homogeneous Markov process with a denumerable state space.

It is well-known that in many cases the matrix of transition probabilities $P(t) = (p_{ij}(t))$ satisfies the backward and forward Kolmogorov differential equations,

$$\left.\begin{aligned}
P'(t) &= QP(t),\\
P'(t) &= P(t)Q,\\
P(0) &= I \text{ (the identity matrix).}
\end{aligned}\right\} \qquad (1)$$

In the above, $Q = (q_{ij})$ is the matrix of infinitesimal transition probabilities, where

$$q_{ij} = p_{ij}'(0) = \lim_{t \downarrow 0} \frac{(p_{ij}(t) - \delta_{ij})}{t}. \tag{2}$$

In recent years several authors have utilized the theory of semigroups of operators to study Markov processes with a denumerable state space. In this approach the process $\mathfrak{M}$ is described by a linear bounded operator P_t on the Banach space $\mathfrak{l}$ to itself, defined by

$$(P_t x)_j = \sum_{k \in \mathfrak{X}} x_k p_{kj}(t), \qquad x \in \mathfrak{l}. \tag{3}$$

Properties (i)–(iv) imply that $\mathcal{S} = \{P_t: t \geq 0\}$ is a *contraction semigroup* of class $(\mathfrak{C}_0)$, i.e., that

(a) $P_t x \geq 0, \quad x \geq 0, \quad t \geq 0;$

(b) $\|P_t x\| \leq \|x\|, \quad x \geq 0, \quad t \geq 0;$

(c) $P_{s+t} = P_s P_t, \quad P_0 = I, \; s,t \geq 0;$

(d) $\lim_{t \downarrow 0} \|(P_t - I)x\| = 0$, for each $x \in \mathfrak{l}$.

If (ii′) holds, (*b*) is replaced by

$$(b')\|P_t x\| = \|x\|, \qquad x \geq 0, \qquad t \geq 0,$$

so that $\mathcal{S}$ is a *transition semigroup*.

The semigroup is uniquely determined by its infinitesimal generator Ω. This linear operator is defined by

$$\Omega x = \lim_{t \downarrow 0} \frac{(P_t - I)x}{t}. \tag{4}$$

The domain of Ω, $\mathfrak{D}(\Omega)$, consists of those elements x for which the strong limit in (4) exists. Since $\mathcal{S}$ has a unique representation (3) in terms of a process $\mathfrak{M}$, we can, from the point of view of functional analysis, identify a given process $\mathfrak{M}$ with the associated semigroup $\mathcal{S}$ of transition operators on the Banach space $\mathfrak{l}$.

Most of the studies in the semigroup theory of Markov processes have been concerned with the existence and uniqueness theory of the Kolmogorov equations,[3, 4, 9] certain pathological processes,[6, 7] and ergodic properties.[1, 8, 10] In this note we wish to consider another application of semigroup theory. In particular, we consider the use of semigroup theory in connection with the method of probability-generating functions. Our primary purpose is to ascertain what properties of a particular process $\mathfrak{M}$ can be deduced from the properties of the semigroup of operators associated with the generating function.

2. Let $X(t)$ be an integer-valued random variable taking values in a denumerable state space $\mathfrak{X}$; and $P_x(t) = Pr(X(t) = x)$, $x \in \mathfrak{X}$. In most applied problems one starts by writing down the forward Kolmogorov equations for the probabilities $P_x(t)$. A standard procedure for solving this infinite system of differential equations is to introduce the *generating function*,

$$F(s, t) = \sum_{x=0}^{\infty} P_x(t) s^x, \qquad |s| \leq 1, \qquad t \geq 0, \tag{5}$$

which transforms the infinite system into a single partial differential equation.[2] To be more precise, the probabilities $P_x(t)$ satisfy the system

$$P_x'(t) = \Omega_1 P_x(t), \qquad x \in X, \qquad t \geq 0, \tag{6}$$

with initial conditions $P_1(0) = 1$, $P_x(0) = 0$, $x \neq 1$, say, where Ω_1 is a difference operator. Application of (5) to (6) yields the partial differential equation,

$$F_t(s, t) = \Omega_2 F(s, t), \qquad |s| \leq 1, \qquad t \geq 0, \tag{7}$$

with initial conditions $F(s, 0) = s$. For simple branching or multiplicative processes, Ω_2 is the differential operator $\varphi(s)\partial/\partial s$, where $\varphi(s)$ is a power series in s convergent in the unit circle. The function $\varphi(s)$ expresses in polynomial form the infinitesimal transition probabilities q_{ij}, since $q_{ij} = i\alpha_{j-i+1}$ and α_n is the coefficient of s^n. For example, in the case of the simple birth-and-death process $\varphi(s) = \lambda s^2 - (\lambda + \mu)s + \mu$, where λ and $\mu > 0$ are the birth and death rates, respectively. In general, since $\sum_j q_{ij} \leq 0$ and $q_{ij} \geq 0$ if $i \neq j$, the only negative coefficient in φ is α_1, and also $\varphi(1) \leq 0$. For more general processes the operator Ω_2 is of the form $\varphi(s)\,\partial/\partial s + \xi(s)$, where the function ξ represents an "input."

3. From the point of view of semigroup theory the difference operator Ω_1 in (6) is the infinitesimal generator of a semigroup of transitions operators of class $(\mathfrak{C}_0)$ on the Banach space $\mathfrak{l}$. The connection between equation (7) and semigroup theory is expressed by the following

THEOREM. *The differential operator Ω_2 in (7) is the infinitesimal generator of a semigroup of transition operators $\mathfrak{IC} = \{T_t : t \geq 0\}$ of class $(\mathfrak{C}_0)$ on any of the following Banach spaces:* (1) $\mathfrak{A}$—*the space of functions analytic within the unit circle and continuous on the boundary;* (2) $\mathfrak{B}$—*the space of all power series whose sum of absolute values of coefficients is convergent; and* (3) $\mathfrak{C}$—*the space of all bounded analytic functions. The domain of Ω_2 includes those functions whose derivative is in the Banach space.*

We first observe that if (5) is the generating function of the probabilities $P_x(t)$ associated with a simple branching or multiplicative process under the initial condition $P_1(0) = 1$, $P_x(0) = 0$, $x \neq 1$, then $F(s, t)$ satisfies the functional equation

$$F(s, t_1 + t_2) = F[F(s, t_1), t_2]. \tag{8}$$

Hence, if the collection of operators $\{T_t\}$ is defined by

$$T_t s = F(s, t), \tag{9}$$

it is easily verified that $T_{t_1+t_2} = T_{t_1}T_{t_2} = 1$, so that $\mathfrak{IC} = \{T_t : t \geq 0\}$ is a semigroup of operators of class $(\mathfrak{C}_0)$. As far as we know, this relationship between semigroups of operators and generating functions was first pointed out by D. G. Kendall.[5]

Let us denote an element of $\mathfrak{A}$ (or $\mathfrak{B}$) by g, where $g(s, t) \equiv F(s, t)$ if and only if $g(s, 0) = s$. If we define the norm of T_t on $\mathfrak{A}$ (or $\mathfrak{C}$) as $\sup_s |g(s, t)|$ for those functions g such that $\|g(\cdot, 0)\| = 1$, then $\|T_t\|_{\mathfrak{A}\ (\text{or } \mathfrak{C})} = 1$; and if we define the norm of T_t on $\mathfrak{B}$ as the sup of the sum of the absolute values of the coefficients of $g(\cdot, t)$ for those functions g such that $\|g(\cdot, 0)\| = 1$, then $\|T_t\|_{\mathfrak{B}} = 1$. Hence $\mathfrak{IC}$ is a transition semigroup of operator on $\mathfrak{A}$, $\mathfrak{B}$, or $\mathfrak{C}$. That $\mathfrak{IC}$ is generated by Ω_2 follows from an application of the Hille-Yosida theorem.[3]

It is of interest to point out the following relations between the spaces $\mathfrak{A}$, $\mathfrak{B}$, and $\mathfrak{C}$: (i) $\mathfrak{B} \subset \mathfrak{A}$, (ii) $\mathfrak{B}$ is isomorphic to I, (iii) $\mathfrak{B}$ is dense in $\mathfrak{A}$ in the $\mathfrak{A}$-norm, but not dense in $\mathfrak{C}$. We also remark that, for processes of the branching or multiplicative type, T_t operates also on the Banach algebra of functions of either $\mathfrak{A}$, $\mathfrak{B}$, or $\mathfrak{C}$.

A generalization of relation (9) for nonmultiplicative processes is given by the following

THEOREM. *If in* (7) $\Omega_2 = \varphi(s)\,\partial/\partial s + \xi(s)$, *then* Ω_2 *is the infinitesimal generator of a semigroup of transition operators* $\mathfrak{IC} = \{T_t : t \geq 0\}$ *of class* $(\mathfrak{C}_0)$ *on the Banach space* $\mathfrak{A}$ (*or* $\mathfrak{B}$), *and*

$$(T_u g(\cdot, t))(s) = g(F(s, u), t)\, exp\left\{\int_0^u \xi(F(s, \tau))d\tau\right\}. \tag{10}$$

We now state several theorems which relate to the spectral properties of the operator Ω_2 and the behavior of the process $\mathfrak{M}$.

THEOREM. *There exists a discrete spectrum of* $\Omega_2 = \varphi(s)\partial/\partial s$ *if and only if the process* $\mathfrak{M}$ *explodes and* $\int^1 \frac{ds}{\varphi(s)}$ *converges.*

THEOREM. *There exists a discrete spectrum of* $\Omega_2 = \varphi(s)\partial/\partial s$ *if the process* $\mathfrak{M}$ *explodes and the explosion takes place exponentially, i.e., the differential probability of explosion is exponential.*

THEOREM. *If the process* $\mathfrak{M}$ *does not explode, zero is in the point spectrum of* $\Omega_2 = \varphi(s)\,\partial/\partial s + \xi(s)$.

THEOREM. *The resolvent spectrum of* $\Omega_2 = \varphi(s)\,\partial/\partial s + \xi(s)$ *contains the negative numbers.*

Proofs of the theorems stated and applications to particular processes, both univariate and bivariate, will be given in a subsequent publication.

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*THE WORD PROBLEM**

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INTRODUCTION; SUMMARY OF RESULTS

Result a. *There is exhibited a group given by a finite number of generators and a finite number of defining relations and having an unsolvable word problem.*

In the present account the finite presentation of a group used for Result a is much simpler in form than that of UG;[1] moreover, the symmetry argument of Theorem III*, Case 2, UG, Part V, is replaced by the very simple Lemma 7 of UG, Part II. The idea behind this change is that the inverses of the group generators relative to which a presentation is given can be taken as an anti-isomorphic copy of the generators. Since in any group $\mathbf{A}^{-1}$ equals $\mathbf{B}^{-1}$ is a consequence of $\mathbf{A}$ equals $\mathbf{B}$, $\mathbf{PAP}^{-1}$ equals $\mathbf{QAQ}^{-1}$ of $\mathbf{AP}^{-1}\mathbf{Q}$ equals $\mathbf{P}^{-1}\mathbf{QA}$—and vice versa—the connection between the old and the new arguments can be seen from the well-known Tietze transformation theorems.[2]

To obtain Result a, we use a certain Thue system of Post,[3] but Result b shows that one may use, instead, any Thue system with an unsolvable word problem.

Result b. *There is explicitly given a recursive mapping, φ, from the set of Thue systems into the set of finite presentations of groups such that the equality of the arbitrary words $\mathbf{A}$ and $\mathbf{B}$ in the Thue system $\mathfrak{T}$ is equivalent to the equality of certain words—recursively specified in terms of $\mathbf{A}$ and $\mathbf{B}$—in the finite group presentation $\varphi(\mathfrak{T})$. Thus if $\mathfrak{T}$ has an unsolvable word problem, so also has $\varphi(\mathfrak{T})$.*[4]

Magnus[5] has shown that any finite presentation of a group consisting of one non-trivial defining relation has a solvable word problem. The group presentation of Result a has an extremely large number of defining relations. A natural question is this: What is the smallest number of defining relations which a finite presentation of a group with unsolvable word problem can have? How long and how "complicated" must these relations be? Result c gives a program for producing, from Thue systems, "simple-looking" finite presentations of groups having unsolvable word problems. Using an unsolvability result of Dana Scott[6] on Thue systems, a result noted by Marshall Hall,[7] and an imbedding theorem for groups of Higman, Neumann, and Neumann,[8] it follows from Result c that one may exhibit a group given by two generators and thirty-two defining relations and having an unsolvable word problem.

Result c. *Let $g_1, g_2, \ldots, g_N$ and $\mathbf{A}_1 = \mathbf{B}_1, \mathbf{A}_2 = \mathbf{B}_2, \ldots, \mathbf{A}_M = \mathbf{B}_M$ be the generators and defining relations of an arbitrary Thue system $\mathfrak{T}$. Let $\mathbf{P}$ by any fixed word of $\mathfrak{T}$. Then $\mathfrak{T}_\mathbf{P}$ is the finite presentation of a group depending on $\mathfrak{T}$ and $\mathbf{P}$ and described as follows:*

The $N + 9$ generators of $\mathfrak{T}_\mathbf{P}$:

$$g_1, g_2, \ldots, g_N;\ q, t_1, t_2, k, a, b, c, d, e.$$

The $6N + M + 13$ non-trivial defining relations of $\mathfrak{T}_\mathbf{P}$:

$$\left.\begin{aligned} q\mathbf{A}_j &= d^j e^j a c^j d^j q \mathbf{B}_j b^j c^j a e^j b^j, \; j = 1, 2, \ldots M \\ qg_i &= g_i q \\ ag_i &= g_i a \\ cg_i &= g_i c \\ eg_i &= g_i e \end{aligned}\right\} i = 1, 2, \ldots, N$$

$$g_i d = d^{M+1} a d^{M+1} g_i \qquad bg_i = g_i b^{M+1} a b^{M+1}$$

$$\left.\begin{aligned} t_u a &= a t_u \\ t_u c &= c t_u \\ t_u d &= d t_u \\ t_u e &= e t_u \end{aligned}\right\} u = 1, 2 \qquad \begin{aligned} ak &= ka \\ bk &= kb \\ ck &= kc \\ ek &= ke \end{aligned}$$

$$t_1 q \mathbf{P} k \mathbf{P}^{-1} q^{-1} t_1^{-1} = t_2 q \mathbf{P} k \mathbf{P}^{-1} q^{-1} t_2^{-1}.$$

For any word $\mathbf{W}$ *of* $\mathfrak{T}$, $\mathbf{W}$ *equals* $\mathbf{P}$ *in* $\mathfrak{T}$ *if and only if*

$$t_1 \mathbf{W} k \mathbf{W}^{-1} t_1^{-1} \text{ equals } t_2 \mathbf{W} k \mathbf{W}^{-1} t_2^{-1}$$

in $\mathfrak{T}_\mathbf{P}$. *Thus, if it is recursively unsolvable to determine for an arbitary word* $\mathbf{W}$ *of* $\mathfrak{T}$ *whether or not* $\mathbf{W}$ *equals* $\mathbf{P}$ *in* $\mathfrak{T}$, *then the word problem for* $\mathfrak{T}_\mathbf{P}$ *is unsolvable.*

Finally we note a very easy direct proof of the unsolvability of the word problem for the finitely generated infinitely related case.

Demonstration of Result a. The Thue system $\mathfrak{T}_1$ given below can be taken to be any Thue system having the form stipulated. The Thue system $\mathfrak{T}_2$, which depends on $\mathfrak{T}_1$, is a finite presentation of a group. We use $\Sigma, \Sigma', \ldots \Gamma, \Gamma', \ldots$ as a variable for words on $\mathfrak{Z}_1$ (i.e., consisting of the symbols of $\mathfrak{Z}_1$) which are of the form $\Delta q_\alpha \Pi$, where Δ and Π are words on $s_1, s_2, \ldots, s_M$ and q_α is $q, q_1, \ldots$ or q_N.

$\mathfrak{T}_1$

$\mathfrak{Z}_1$: $s_1, s_2, \ldots, s_M; \quad q_1, q_2, \ldots, q_N, q;$

$\mathfrak{U}_1$: $\Sigma_1 = \Gamma_1, \Sigma_2 = \Gamma_2, \ldots, \Sigma_P = \Gamma_P.$

$\mathfrak{T}_2$

$\mathfrak{Z}_2$: All symbols of $\mathfrak{Z}_1$; $t_1, t_2, k, x, y;$ $l_\iota, r_\iota, \iota = 1, 2, \ldots, P;$ Each of the above symbols with superscript -1 added.

$\mathfrak{U}_2$: Where $\iota = 1, 2, \ldots, P$, $\alpha = 1, 2,$ and $\beta = 1, 2, \ldots, M$, the following:

2.1 $\quad \Sigma_\iota = l_\iota \Gamma r_\iota$

2.2	$s_\beta l_\iota = y l_\iota y s_\beta$	2.6	$r_\iota s_\beta = s_\beta x r_\iota x$
2.3	$s_\beta y = y y s_\beta$	2.7	$x s_\beta = s_\beta x x$
2.4	$t_\alpha l_\iota = l_\iota t_\alpha$	2.8	$r_\iota k = k r_\iota$
2.5	$t_\alpha y = y t_\alpha$	2.9	$xk = kx$

2.10 $\quad kq^{-1}t_1^{-1}t_2 q = q^{-1}t_1^{-1}t_2 qk.$

Where $\mathbf{a}$ and $\mathbf{a}^{-1}$ are symbols of $\mathfrak{Z}_2$ the following:

2.11 $\quad \mathbf{a}^{-1}\mathbf{a} = 1$ (where 1 is the empty word)

2.12 $\quad \mathbf{a}\mathbf{a}^{-1} = 1.$

A proof of $\mathbf{A}/\mathbf{B}$ *in* $\mathfrak{T}_i$, $i = 1, 2,$ is a finite sequence of words on $\mathfrak{Z}_i$ called *steps*, say $\mathbf{C}_1, \mathbf{C}_2, \ldots, \mathbf{C}_n$, such that $\mathbf{C}_1$ is $\mathbf{A}$ and $\mathbf{C}_n$ is $\mathbf{B}$; each $\mathbf{C}_\nu$ and $\mathbf{C}_{\nu+1}$, $\nu = 1, 2, \ldots, n-1$, are $\mathbf{PDQ}$ and $\mathbf{PEQ}$ where $\mathbf{D} = \mathbf{E}$ is a rule of $\mathfrak{U}_i$ for some $\mathbf{P}$ and $\mathbf{Q}$, possibly void.[9] We abbreviate "there is a proof of $\mathbf{A}/\mathbf{B}$ in $\mathfrak{T}_i$" to $\mathbf{A} \vdash_i \mathbf{B}$.

MAIN THEOREM. *For any choice of* $\mathfrak{T}_1$ *and* Σ, $\Sigma \vdash_1 q$ *if and only if*

$$t_1 \Sigma k \Sigma^{-1} t_1^{-1} \vdash_2 t_2 \Sigma k \Sigma^{-1} t_2^{-1}.$$

From Post[3] we have that for a certain choice of $\mathfrak{T}_1$ it is unsolvable to determine for arbitrary Σ on $\mathfrak{Z}_1$ whether or not $\Sigma \vdash_1 q$. Thus the Main Theorem implies Result a. The "only if" part of the Main Theorem is easy. Let Ξ (Let Ω) be a variable for words on y, y^{-1}, and the symbols l_ν, l_ν^{-1} of $\mathfrak{Z}_2$ (on x, x^{-1}, and the symbols r_ν, r_ν^{-1} of $\mathfrak{Z}_2$). Suppose $\Sigma \vdash_1 q$. Then for some Ξ and Ω, $\Sigma \vdash_2 \Xi q \Omega$. Consequently—noting $t_1 q k q^{-1} t^{-1} \vdash_2 t_2 q k q^{-1} t_2^{-1}$—we have $t_1 \Sigma k \Sigma^{-1} t_1^{-1} \vdash_2 t_2 \Sigma k \Sigma^{-1} t_2^{-1}$.

To show the Main Theorem in the non-trivial direction, we first stipulate three additional group presentations, $\mathfrak{T}_3$, $\mathfrak{T}_4$, and $\mathfrak{T}_5$. We define $\mathfrak{Z}_3$ to be $\mathfrak{Z}_2$; $\mathfrak{U}_3$ to be $\mathfrak{U}_2$ with each $\mathbf{a}k = k\mathbf{a}$ of $\mathfrak{U}_{2.8}$ or $\mathfrak{U}_{2.9}$ replaced by $\mathbf{a}k = k$ and $\mathfrak{U}_{2.10}$ replaced by $t_1 q k = t_2 q k$. We define $\mathfrak{Z}_4$ to be $\mathfrak{Z}_3$; $\mathfrak{U}_4$ to be $\mathfrak{U}_3$ with each $\mathbf{a}t_\nu = t_\nu \mathbf{a}$ of $\mathfrak{U}_{2.4}$ or $\mathfrak{U}_{2.5}$ replaced by $t_\nu \mathbf{a} = t_\nu$ and $t_1 q k = t_2 q k$ excluded. We define $\mathfrak{Z}_5$ to $\mathfrak{Z}_4$ with k, k^{-1}, t_ν, t_ν^{-1} excluded; $\mathfrak{U}_5$ to be $\mathfrak{U}_4$ with the rules containing k, k^{-1}, t_ν, t_ν^{-1} excluded. Let $\mathbf{A} \vdash_i \mathbf{B}$, $i = 1, 2, \ldots, 5$, mean $\mathbf{A} \vdash_i \mathbf{B}$ with no occurrences of q^{-1}, q_0^{-1}, $\ldots$, q_N^{-1} in any step. Then the plan of the argument is given below in the first column; the first statement in each brace implies the second.

$t_1 \Sigma k \Sigma^{-1} t_1^{-1} \vdash_2 t_2 \Sigma k \Sigma^{-1} t_2^{-1}$ } [10] Cf. UG, Part II, Sec. 5–7, especially Lemma 7.

$t_1 \Sigma k \Sigma^{-1} t_1^{-1} \vdash_{2k} t_2 \Sigma k \Sigma^{-1} t_2^{-1}$ } Roughly speaking, erase all symbols right of k in each step of the $\mathfrak{T}_2$ proof.

$t_1 \Sigma k \vdash_{3k} t_2 \Sigma k$ } Cf. UG, Part II, Sec. 5–7, especially Lemma 7.

$t_1 \Sigma k \vdash_{3k} t_1 t_2 \Sigma k$ } See i below.

$t_1 \Sigma k \vdash_{4k} t_1 q k$ } See ii below.

$\Sigma \vdash_5 \Xi q \Omega$ for some Ξ and Ω

$\Sigma \vdash_5 \Xi q \Omega$ } See iii below.

$\Sigma \vdash_{5q} \Xi q \Omega$ } Cf. UG, Part VI, Sec. 20.

$\Sigma \vdash_{5q^2} \Xi q \Omega$ } Erase the symbols l_ν, or r_ν, l_ν^{-1}, r_ν^{-1}, everywhere in the $\mathfrak{T}_5$ proof.

$\Sigma \vdash_1 q$

(i) Omit from the $\mathfrak{T}_3$ proof all steps following the first application of $t_1 q k = t_2 q k$; then erase from each step remaining all symbols left of t_1, all right of k. (ii) It is sufficient to show $\mathbf{M}_i \vdash_5 \Xi' \mathbf{M}_{i+1} \Omega'$ for some Ξ' and Ω', where $t_1 \mathbf{M}_i k$ is the ith step of the $\mathfrak{T}_4$ proof. (iii) Cf. UG, Part II, Sec. 5–7, and Part III. But the argument of Part III can be replaced by an almost trivial one using the techniques of UG, Part VI, Sec. 20 and 21 and the fact that the set of words l_ν, l_ν^{-1} (or words r_ν, r_ν^{-1}) is *independent.*[11]

Demonstration of Result b. Throughout this section, $\mathfrak{T}_0$, $\mathfrak{T}_*$, $\mathfrak{T}_{(\mathbf{W})}$, are Thue systems depending on the arbitrary Thue system $\mathfrak{T}$ (with symbols $\mathfrak{Z}$ and rules $\mathfrak{U}$)—$\mathfrak{T}_{(\mathbf{W})}$ depending on the word $\mathbf{W}$ on $\mathfrak{Z}$ as well. Let $\mathfrak{Z}_0$ contain p, v and $\mathbf{a}$, $\mathring{\mathbf{a}}$ for each $\mathbf{a}$ of $\mathfrak{Z}$ and let $\mathfrak{U}_0$ contain the rules of $\mathfrak{U}$ and $\mathbf{a}\mathring{\mathbf{b}} = \mathring{\mathbf{b}}\mathbf{a}$, $p\mathring{\mathbf{a}}\mathbf{a} = \mathring{\mathbf{a}}p$, $\mathring{\mathbf{a}}pv = pv$ for each $\mathbf{a}$ of $\mathfrak{Z}$. *Let* $\mathbf{W}$ *and* $\mathbf{V}$ *be words on* $\mathfrak{Z}$, $\mathbf{V}_0$ *the word obtained from* $\mathbf{V}$ *by replacing* $\mathbf{a}$ *by* $\mathring{\mathbf{a}}$. *Then* $p\mathbf{V}_0\mathbf{W}v \vdash_{\mathfrak{T}_0} pv$ *if and only if* $\mathbf{W} \vdash_{\mathfrak{T}} \mathbf{V}$. Let $\mathfrak{Z}_*$ consist of $\mathfrak{Z}$ and q_1; $\mathfrak{U}$ con-

tains $q_1\mathbf{A} = q_1\mathbf{B}$, $q_1\mathbf{a} = \mathbf{a}q_1$ for each $\mathbf{A} = \mathbf{B}$ of $\mathfrak{U}$ and $\mathbf{a}$ of $\mathfrak{Z}$. Then *for any* $\mathbf{W}$ *and* $\mathbf{V}$ *on* $\mathfrak{Z}$, $q_1\mathbf{W} \vdash_{\mathfrak{T}_*} q_1\mathbf{V}$ *if and only if* $\mathbf{W} \vdash_{\mathfrak{T}} \mathbf{V}$. Let $\mathfrak{Z}_{(\mathbf{W})}$ consist of $\mathfrak{Z}$ and q; $\mathfrak{U}_{(\mathbf{W})}$, of $\mathfrak{U}$ and $\mathbf{W} = q$. Then, *for any* $\mathbf{V}$ *on* $\mathfrak{Z}$, $\mathbf{V} \vdash_{\mathfrak{T}_{(\mathbf{W})}} q$ *if and only if* $\mathbf{V} \vdash_{\mathfrak{T}} \mathbf{W}$. Thus for any $\mathfrak{T}$ and words $\mathbf{A}$ and $\mathbf{B}$ on $\mathfrak{Z}$ the following statements are equivalent: $\mathbf{A} \vdash_{\mathfrak{T}} \mathbf{B}$; $p\mathbf{B}_0\mathbf{A}v \vdash_{\mathfrak{T}_c} pv$; $q_1p\mathbf{B}_0\mathbf{A}v \vdash_{\mathfrak{T}_{c*}} q_1pv$; $q_1p\mathbf{B}_0\mathbf{A}v \vdash_{\mathfrak{T}_{c*(q_1pv)}} q$. This shows Result b since $\mathfrak{T}_{c*(q_1pv)}$ has the form of $\mathfrak{T}_1$ used in the demonstration of Result a.

Demonstration of Result c. The argument is a revision of that for Result a. The main points are: (1) the proof of Result a is valid if the q of $\mathfrak{U}_{2.10}$ is interpreted as some fixed special word of $\mathfrak{T}_1$ and $\mathfrak{U}_{2.10}$ rewritten in the symmetric form of the last relation listed in Result c; (2) if $\mathfrak{T}$ is any Thue system then the $\mathfrak{T}_*$ used for Result b has the form of $\mathfrak{T}_1$ used for Result a; (3) the set of words c^je^j, $j = \pm 1, \pm 2, \ldots, \pm M$ is *independent*,[11] as is the set $c^{\pm 1}$, $e^{\pm 1}$, $d^{\pm 1}$, $b^{M+1}ab^{M+1}$, $b^jc^jae^jb^j$ $j = 0, \pm 1, \ldots, \pm M$.

Result c *and* $\mathfrak{T}_0$. Starting with an arbitrary system $'\mathfrak{T}$, the Thue system $'\mathfrak{T}_0$ (see Result b) can be identified with the $\mathfrak{T}$ of Result c. Thus if $'\mathfrak{T}$ has an unsolvable word problem, so has $'\mathfrak{T}_{0pv}$. Using this construction in connection with Scott's result yields a forty defining relation group with unsolvable word problem that can actually be written down in a few minutes' time.[12]

The Word Problem for the Finitely Generated Infinitely Related Case.[13] Where S is any set of ordered pairs of positive integers, let $\mathfrak{T}_S$ be the following group presentation.

$$\begin{array}{ll} \mathfrak{Z}_S: & z, x_1, x_2, q \\ \mathfrak{U}_S: & z^m x_1^n q x_1^{-n} = x_2^n q x_2^{-n} \qquad \text{for each } (m, n) \text{ of } S. \\ & z = 1 \end{array}$$

THEOREM. $x_1^n q x_1^{-n} \vdash_{\mathfrak{T}_S} x_2^n q x_2^{-n}$ *if and only if there is an* m *such that* (m, n) *is a member of* S.

The theorem implies the desired unsolvability result, for we may take any well-known S such that (1) there is a recursive procedure to determine for an arbitrary pair of positive integers, (m, n), whether or not (m, n) is a member of S; (2) there is no recursive procedure to determine for arbitrary n whether or not there is an m such that (m, n) is a member of S.[14]

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[1] W. W. Boone, "Certain Simple Unsolvable Problems of Group Theory," *Koninkl. Ned. Akad. Wetenschap.*, Ser. A, Part I, **57**, 231–237, 1954; Part II, pp. 492–497; Part III, **58**, 252–256, 1955; Part IV, pp. 571–577; Part V, **60**, 22–27, 1957; Part VI, pp. 227–232. In Part V, last line of p. 24, read "and 7" after "6" and "their" for "its." We have attempted to make the present note as self-contained as possible.

[2] The relation of our work to P. S. Novikov, *On the Algorithmic Unsolvability of the Word Problem in Group Theory* (Trudy Mat. Inst. Steklov, No. 44 [1955]) (in Russian), is still essentially unknown to us (see A. A. Markov, *Math. Rev.*, **17**, 706; an A.M.S. translation of Novikov by K. A. Hirsch is in preparation). Through J. L. Britton we do know that Novikov uses the symmetry argument of UG, Part V. At the British Mathematical Colloquium, Nottingham, September, 1957, Britton announced a new proof of the unsolvability of the word problem based to some extent on Novikov's proof. Our Results b and c were presented at this colloquium. Result a—using the $\mathfrak{T}_2$ given below but with the old symmetry argument—on a Fulbright Inter-Foundation Lectureship tour in the United Kingdom in May, 1957.

[3] E. L. Post, *J. Symb. Logic*, **12**, 1–11, 1947.

[4] In the sense of Post, the word problem for $\mathfrak{T}$ is *reducible to that for* $\varphi(\mathfrak{T})$.

[5] W. Magnus, *Math. Annalen*, **106**, 295–307, 1932.

[6] Dana Scott, abstract, *J. Symb. Logic*, **21**, 111–112, 1956.

[7] Marshall Hall, *J. Symb. Logic*, **14**, 115–118, 1949.

[8] G. Higman, B. H. Neumann, and H. Neumann, *J. London Math. Soc.*, **24**, 247–254, 1949.

[9] As defined, UG, Part I, p. 234.

[10] Also provable by Theorem I of Higman, Neumann, and Neumann, *op. cit.*, as pointed out to us by Higman.

[11] The set of words $\mathbf{A}_1, \mathbf{A}_2, \ldots, \mathbf{A}_K$ on 3 is *not* independent if there is a product of the **A**'s, with no adjacent **A**'s inverses of each other, which equals 1 in the free group on 3.

[12] The 32 defining relation group mentioned above has one relation which is astronomical in length.

[13] Included in a report filed with the National Science Foundation on contract G-1974, May 28, 1956—but in a form more akin to UG, Parts V–VI, than Result a as presently shown (cf. W. Craig, *J. Symb. Logic*, **18**, 30–32, 1953, and B. H. Neumann, *J. London Math. Soc.*, **12**, 125, Theorem (13), 1937).

[14] For related material see W. W. Boone, abstract, *Bull. A.M.S.*, **62**, 148, 1956.

NON-ADDITIVE FUNCTORS, THEIR DERIVED FUNCTORS, AND THE SUSPENSION HOMOMORPHISM

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1. *Derived Functors of Non-additive Functors.*—Let T be a (covariant) functor of modules over a ring Λ to modules over a ring Λ'. If T is additive its derived functors have been defined by Cartan-Eilenberg.[2] Additivity is used to show that T applied to a chain homotopy again gives a chain homotopy (cf.[2] IV, 5, and V, 3). Using *FD*-complexes[4] instead of chain complexes, we define left derived functors for arbitrary functors T.

1.1. *Definition.—A projective FD-resolution of type n* of the module M is an *FD*-module P such that (i) $P_j = 0$ for $j < n$, (ii) P_j is projective for all j, (iii) $H_n(P) = M$, $H_j(P) = 0$ for $j \neq n$.

Passing from an *FD*-module to its normal(ized) chain module establishes a 1 to 1 correspondence (up to natural equivalences) between *FD*-modules, *FD*-maps, *FD*-homotopies and chain modules, chain maps, chain homotopies.[3] In particular, it establishes such a correspondence between projective *FD*-resolutions P of type n of M and chain modules C for which $C_j = 0$ for $j < n$ and

$$0 \leftarrow M = H_n(C) \leftarrow C_n \leftarrow C_{n+1} \leftarrow C_{n+2} \leftarrow \ldots \tag{1.2}$$

is an ordinary projective resolution[2] of M. From the corresponding properties of ordinary resolutions it follows: Every module has a projective *FD*-resolution of any given type n. If $f\colon M \to M'$ is a homomorphism and P,P' are projective *FD*-resolutions of type n of M,M' resp., then there exists an *FD*-map $F\colon P \to P'$ such that $F_*\colon H_n(P) \to H_n(P')$ equals f. Moreover, if F' is another such map, then F,F' are *FD*-homotopic (cf. Cartan-Eilenberg,[2] V, 1).

Now let T be an arbitrary functor from modules to modules. Its prolongation[3] to FD-modules (defined by applying T is every dimension and also to the face- and degeneracy operators) preserves homotopy, i.e., $F \simeq F'$ implies $TF \simeq TF'$ for all FD-maps F,F', even if T is not additive.[3] Hence it follows, as in Cartan-Eilenberg[2] (V, 3), that $H_q(TP)$ depends only on M and n (not on P), and $(TF)_*$: $H_q(TP) \to H_q(TP')$ depends only on f and n. We denote $H_q(TP)$, $(TF)_{*q}$ by $L_q^{(n)}TM$, $L_q^{(n)}Tf$ resp., and call $L_q^{(n)}T$ the *qth left derived functor of T of type n*.

If T is additive, $L_q^{(n)}T$ coincides with the left derived functor $L_{q-n}T$ as defined in Cartan-Eilenberg.[2] For arbitrary T we shall exhibit a natural transformation σ: $L_q^{(n)}T \to L_{q+1}^{(n+1)}T$, the *suspension* (cf. (2.4)). σ is an isomorphism for additive T, but not in general.

1.3. *Examples.*—Let T,T' be the functors from abelian groups to Λ-modules defined by $TM = N \otimes Z(M)$, $T'M = N \otimes SA(M)$, where N is a fixed Λ-module, $Z(M)$ is the group ring, and $SA(M)$ is the symmetric algebra of the abelian group M. Then $L_q^{(n)}TM$ for all n, and $L_q^{(n)}T'M$ for $n > 0$ are the Eilenberg-Mac Lane modules $H_q(M, n; N)$.[4] We do not know how to answer the interesting question as to the nature of the derived functors of the Γ-functor of Eilenberg-Mac Lane.[5]

2. *The Suspension.*—Define the *cone over an FD module K* to be the FD-module CK given by

$$\left.\begin{aligned}
&(CK)_q = K_q + K_{q-1} + K_{q-2} + \ldots + K_0,\\
&\partial_i(a_q, a_{q-1}, \ldots, a_0) = (\partial_i a_q, \partial_{i-1}a_{q-1}, \ldots, \partial_1 a_{q-i+1}, \partial_0 a_{q-i} + a_{q-i-1}, a_{q-i-2}, \ldots, a_0),\ i < q,\\
&\partial_q(a_q, a_{q-1}, \ldots, a_0) = (\partial_q a_q, \partial_{q-1}a_{q-1}, \ldots, \partial_1 a_1),\\
&s_i(a_q, a_{q-1}, \ldots, a_0) = (s_i a_q, s_{i-1}a_{q-1}, \ldots, s_0 a_{q-i}, 0, a_{q-i-1}, \ldots, a_0),
\end{aligned}\right\} \quad (2.1)$$

where ∂_i and s_i are the face- and degeneracy operators. There is a natural injection i: $K \to CK$, and the FD-module $SK = CK/iK$ is called the *suspension* of K. The exact sequence

$$0 \to K \xrightarrow{i} CK \xrightarrow{p} SK \to 0 \quad (2.2)$$

splits if we disregard the FD-structures, i.e., $(CK)_q \cong K_q + (SK)_q$ for all q. CK is of the same homotopy type as the trivial complex 0 (notation: $CK \equiv 0$), hence $H_q(K) \cong H_{q+1}(SK)$. If P is a projective resolution of type n, then SP is a projective resolution of type $n + 1$ of the same module.

Let T be a functor from modules to modules prolonged to FD-modules as above, and assume $T(0) = 0$. Then $(Tp)(Ti) = T(pi) = 0$, i.e., $im(Ti) \subset ker(Tp)$, and we have $(Ti)_*$: $H_q(TK) \to H_q(ker(Tp))$. Since (2.2) splits, Tp is an epimorphism, hence $TSK \cong TCK/ker(Tp)$. The boundary homomorphism Δ: $H_{q+1}(TSK) \to H_q(ker(Tp))$ is an isomorphism because $CK \equiv 0$ implies $TCK \equiv T(0) = 0$ (T preserves homotopy). So we can form the *suspension homomorphism*,

$$\sigma = \Delta^{-1}(Ti)_*\colon\ H_q(TK) \to H_{q+1}(TSK). \quad (2.3)$$

In particular, if $K = P$ is a projective FD-resolution of type n of M, this gives a natural transformation,

$$\sigma_q^{(n)}(M)\colon\ L_q^{(n)}TM \to L_{q+1}^{(n+1)}TM. \quad (2.4)$$

If T does not satisfy $T(0) = 0$, then $TM = T'M + M_0$, where $T'(0) = 0$, and

M_0 is a fixed module. In order to apply our constructions, replace T by the functor T'.

3. *Properties of the Suspension.*—Eilenberg and Mac Lane[5] defined the kth cross-effect functor of a functor T with $T(0) = 0$. These functors, also denoted by T, satisfy

$$T(M_1 + M_2 + \ldots + M_r) = \sum_{1 \leq i_1 < i_2 < \ldots < i_k \leq r} T(M_{i_1} | M_{i_2} | \ldots | M_{i_k}). \quad (3.1)$$

Letting all variables in the kth cross-effect be equal gives a functor in one variable,

$$T_{[k]}M = T(M|M|\ldots M) \qquad (k \text{ variables}). \quad (3.2)$$

Let $\sum_k M$ denote the direct sum of k copies of M. Define α_i': $\sum_k M \to \sum_{k-1} M$ and β_i': $\sum_{k-1} M \to \sum_k M$ for $0 < i < k$ by

$$\left.\begin{aligned} \alpha_i'(m_1, m_2, \ldots, m_k) &= (m_1, \ldots, m_{i-1}, m_i + m_{i+1}, m_{i+2}, \ldots, m_k) \\ \beta_i'(m_1, m_2, \ldots, m_{k-1}) &= m_i, \ldots, m_{i-1}, m_i, m_i, m_{i+1}, \ldots, m_{k-1}). \end{aligned}\right\} \quad (3.3)$$

By restriction and projection (cf. 3.1) we obtain from $T\alpha_i'$ and $T\beta_i'$ the natural transformations

$$\begin{aligned} &\alpha_i\colon\ T_{[k]}M \to T_{[k-1]}M \\ &\beta_i\colon\ T_{[k-1]}M \to T_{[k]}M \end{aligned} \qquad 0 < i < k. \quad (3.4)$$

α_i and β_i are prolonged to FD-modules by applying them in every dimension.

Let K be an FD-module. The elements in the image of α_{i*}: $H(T(K|K)) \to H(TK)$ resp. in the kernel of β_{i*}: $H(TK) \to H(T(K|K))$ are called *decomposable* resp. *primitive*.

3.5 PROPOSITION. *The decomposable elements of $H(TK)$ lie in the kernel of the suspension σ: $H(TK) \to H(TSK)$. The image of σ consists of primitive elements of $H(TSK)$.*

To obtain further results on the suspension (in particular a partial inverse of 3.5: cf. 3.9) we consider the sequence

$$TK = T_{[1]}K \xleftarrow{d_1} T_{[2]}K \xleftarrow{d_2} \ldots \xleftarrow{d_{k-1}} T_{[k]}K \xleftarrow{d_k} \ldots, \quad (3.6)$$

where $d = d_k = \sum_{j=1}^{k} (-1)^j \alpha_j$. Then $dd = 0$, and $\pm d$, together with the "internal" differential $\partial = \sum (-1)^j \partial_j$ of $T_{[k]}K$, turns $\mathfrak{T}K = \sum_{k=1}^{\infty} T_{[k]}K$ into a double complex. If T stands for "group ring" or "symmetric algebra" (cf. 1.3) $\mathfrak{T}K$ is very much like the bar construction.[4]

3.7. THEOREM. *There is a natural isomorphism $H_{q+1}(TSK) \cong H_q(\mathfrak{T}K)$ which transforms the suspension σ: $H_q(TK) \to H_{q+1}(TSK)$ into the map ι_*: $H_q(TK) \to H_q(\mathfrak{T}K)$ induced by the inclusion ι: $TK \to \mathfrak{T}K$.* ($H(\mathfrak{T}K)$ is the homology of the single complex associated with the double complex $\mathfrak{T}K$; cf. Cartan-Eilenberg,[2] IV, 4.)

There are two spectral sequences associated with the double complex $\mathfrak{T}K$ (cf. Cartan-Eilenberg,[2] XV, 6). One of them can be used to prove 3.7, the other gives

3.8. COROLLARY. *There is a spectral sequence E^r such that E^1 together with its differential d^1 is the (single) complex*

$$H(TK) \xleftarrow{d_*} H(T_{[2]}K) \xleftarrow{d_*} \ldots \xleftarrow{d_*} H(T_{[k]}K) \xleftarrow{d_*} \ldots,$$

and E^∞ is the graded module associated with a certain filtration of $H(TSK)$.

It follows from a generalization of the Eilenberg-Zilber theorem[5] by P. Cartier that $H_q(T_{[k]}K) = 0$ for $q < kn$ if (A_n): $K_q = 0$ for $q < n$. Using Dold,[3] Section 3, one can replace the hypothesis (A_n) by (B_n): K is a projective FD-module over a hereditary ring and $H_q(K) = 0$ for $q < n$. By standard arguments on spectral sequences one then obtains from 3.8

3.9. COROLLARY. *If K satisfies (A_n) or (B_n) then there is an exact sequence*

$$H_q(T_{[2]}K) \xrightarrow{\alpha_{1*}} H_q(TK) \xrightarrow{\sigma} H_{q+1}(TSK) \xrightarrow{\beta} H_{q-1}(T_{[2]}K) \xrightarrow{\alpha_{1*}} H_{q-1}(TK) \quad (3.10)$$

for $q \leqq 3n$; in particular, σ: $H_q(TK) \to H_{q+1}(TSK)$ is an isomorphism for $q < 2n$ and an epimorphism for $q = 2n$.

It can be shown that the homomorphism β in (3.10) is the composition of β_{1*}: $H_{q+1}(TSK) \to H_{q+1}(T_{[2]}SK)$ and an isomorphism $H_{q+1}(T_{[2]}SK) \cong H_{q-1}(T_{[2]}K)$. If T is a quadratic functor, i.e., $T(M_1|M_2|M_3) = 0$ for all M_1, M_2, M_3, there is an exact sequence (3.10) without any assumption on K and without restriction on q.

All results on the suspension have obvious applications to the left derived functors of T. Corollary 3.9 gives, in particular,

$$\sigma\colon\ L_q^{(n)}TM \cong L_{q+1}^{(n+1)}\ TM \qquad \text{for } q < 2n. \quad (3.11)$$

There is a "dual" to the construction (3.6) which resembles the cobar construction.[1] Together with the details about the preceding results, it will appear elsewhere.

[1] J. F. Adams, "On the Cobar Construction," these PROCEEDINGS, **42**, 409–412, 1956.

[2] H. Cartan and S. Eilenberg, *Homological Algebra* (Princeton, N. J., Princeton University Press, 1956).

[3] A. Dold, "Homology of Symmetric Products and Other Functors of Complexes," to appear in *Ann. Math.*, **68**, 1958.

[4] S. Eilenberg and S. Mac Lane, "On the Groups $H(\pi, n)$. I," *Ann. Math.*, **58**, 55–106, 1953.

[5] S. Eilenberg and S. Mac Lane, "On the Groups $H(\pi, n)$. II," *ibid.*, **60**, 49–139, 1954.

DIFFERENTIABLE MAPPINGS IN THE SCHOENFLIES PROBLEM

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1. *Introduction.*—The recent remarkable contributions of Mazur, of Princeton University, to the topological theory of the Schoenflies problem lead naturally to questions of fundamental importance in the theory of differentiable mappings. In particular, if the hypothesis of the Schoenflies Theorem is stated in terms of regular differentiable mappings of class C^m, $m > 0$, can the conclusion be stated in terms of regular differentiable mappings of the same class? This paper presents an outline of an answer to this question. It shows how to develop a differential theory corresponding to Mazur's process.[1]

An abstract r-manifold Σ_r, $r > 1$ of class C^m is understood in the usual sense.[2,3]

We do not require that Σ_r be connected. Given manifolds Σ_r and Σ_r' with C^m-differentiable structures, a homeomorphism ϕ of Σ_r onto Σ_r' which is also a regular differentiable mapping of class C^m, will be called a *C^m-diffeomorphism* (abbr. C^m-diff.).

C_0^m-diffeomorphisms: Let Σ_r and Σ_r' be r-manifolds of class C^m, $m > 0$. Let p and p' be points, respectively, of Σ_r and Σ_r'. Then $\Sigma_r - p$ and $\Sigma_r' - p$ are r-manifolds which possess C^m-structures derived from those of Σ_r and Σ_r'. A homeomorphism Λ of Σ_r onto Σ_r' will be called a C_0^m-diff., if for some $p \in \Sigma_r$ and $p' \in \Sigma_r'$, $\Lambda(p) = p'$, and if the restriction of Λ to $\Sigma_r - p$ is a C^m-diff. of $\Sigma_r - p$ onto $\Sigma_r' - p$. In case Λ is not a C^m-diff. of Σ_r onto Σ_r', we term p *exceptional* under Λ.

Let $n > 1$ be a fixed integer. Let E and $\mathfrak{E}$ be two Euclidean n-spaces. We shall be concerned with an $(n-1)$-sphere S_{n-1} of unit radius in E, and a regular, compact $(n-1)$-manifold $\mathfrak{M}_{n-1}$ in $\mathfrak{E}$ with a C^m-structure, $m > 0$, derived from that of $\mathfrak{E}$. Let $\mathfrak{M}_{n-1}$ be the image of S_{n-1} under a C^m-diff. ϕ. Let $J\ S_{n-1}$ and $J\ \mathfrak{M}_{n-1}$ be respectively the closures of the interiors of S_{n-1} and $\mathfrak{M}_{n-1}$ in E and $\mathfrak{E}$. In referring presently to a C_0^m-diff. of a neighborhood of $J\ S_{n-1}$, we mean a C_0^m-diff. in which the exceptional point (if any exists) is on the interior of S_{n-1}. The principal theorem follows.

THEOREM 1.1. *Given a C^m-diff. ϕ, $m > 0$, of S_{n-1} onto $\mathfrak{M}_{n-1}$ there exists a C_0^m-diff. Λ_ϕ of some open neighborhood of $J\ S_{n-1}$, relative to E, onto some open neighborhood of $J\ \mathfrak{M}_{n-1}$, relative to $\mathfrak{E}$, such that $\Lambda_\phi \mid S_{n-1} = \phi$.*

There are indications that this theorem would be false if C_0^m were replaced by C^m in its statement, at least false for some integers n.[4]

2. *Composite n-Manifolds.*—Let M and $\mathfrak{M}$ be two n-manifolds abstractly given, without points in common. Let W and $\mathfrak{W}$ be fixed open subsets of M and $\mathfrak{M}$, respectively, and μ a homeomorphism of W onto $\mathfrak{W}$. Let each point $p \in W$ be identified with its image $\mu(p)$ to form an identified point $[p:\mu(p)]$. Subject to this identification, let Σ be the ensemble of *all* points of M and $\mathfrak{M}$.

The π-mappings π, π_1, π_2: To each point $p \in M$ corresponds a point $\pi(p) \in \Sigma$, represented by p if $p \notin W$, and by $[p:\mu(p)]$ if $p \in W$. To each point $q \in \mathfrak{M}$ corresponds a point $\pi(q) \in \Sigma$, represented by q if $q \notin \mathfrak{W}$, and by $[\mu^{-1}(q):q]$, if $q \in \mathfrak{W}$. Set $\pi_1 = \pi \mid M$, $\pi_2 = \pi \mid \mathfrak{M}$. The mappings π_1 and π_2 are biunique, but not in general onto Σ; the mapping π of $M \cup \mathfrak{M}$ (points unidentified) is onto Σ, but not in general biunique. We write

$$\Sigma = [M, \mathfrak{M}, \mu, W, \mathfrak{W}],$$

and term this a *canonical* representation of Σ.

Let Σ be topologized as follows. Let X and $\mathfrak{X}$ be arbitrary open sets of M and $\mathfrak{M}$, respectively. Let Φ be the ensemble of subsets of Σ which contains all subsets $\pi_1(X)$ and $\pi_2(\mathfrak{X})$ and is closed with respect to finite intersections of its sets. An open set of Σ shall be the union of an arbitrary collection of sets of Φ. So topologized, Σ is not necessarily a Hausdorf space or a manifold. It is, however, if Condition (α) is satisfied (as we assume). Let $\beta_M W$ denote the boundary of W relative to M, and $\beta_{\mathfrak{M}} \mathfrak{W}$ the boundary of $\mathfrak{W}$, relative to $\mathfrak{M}$.

Condition (α). For arbitrary points $p \in \beta_M W$ and $q \in \beta_{\mathfrak{M}} \mathfrak{W}$ there shall exist neighborhoods N_p and N_q, relative to M and $\mathfrak{M}$, respectively, such that

$$\pi_1(N_p \cap W) \cap \pi_2(\mathfrak{N}_q \cap \mathfrak{W}) = 0.$$

μ-Represented subsets of Σ: Let A and $\mathfrak{A}$ be subsets, respectively, of M and $\mathfrak{M}$, and set

$$\pi_1(A) \cup \pi_2(\mathfrak{A}) = [A, \mathfrak{A}, \Sigma].$$

This subset of Σ will be said to be μ-represented if

$$\mu(A \cap W) = \mathfrak{A} \cap \mathfrak{W}.$$

Two μ-represented subsets of Σ, $[A, \mathfrak{A}, \Sigma]$ and $[B, \mathfrak{B}, \Sigma]$, have a μ-represented intersection and union

$$[A \cap B, \mathfrak{A} \cup \mathfrak{B}, \Sigma], \qquad [A \cup B, \mathfrak{A} \cup \mathfrak{B}, \Sigma].$$

This extends to infinite unions. The complement of a μ-represented subset $[A, \mathfrak{A}, \Sigma]$ of Σ is a μ-represented subset $[M - A, \mathfrak{M} - \mathfrak{A}, \Sigma]$. The algebra of these subsets is basic.

A C^m-structure on Σ: Suppose M and $\mathfrak{M}$ have C^m-structures and that the mapping μ is a C^m-diff. Let $[F]$ and $[\mathfrak{F}]$ be, respectively, sets of local representations of M and $\mathfrak{M}$, where $F \in [F]$ maps an open Euclidean region U onto a domain of coordinates $X \subset M$, and $\mathfrak{F} \in [\mathfrak{F}]$ maps an open Euclidean region $\mathfrak{U}$ onto a domain of coordinates $\mathfrak{X} \subset \mathfrak{M}$. We suppose that a C^m-structure is thereby defined on M and $\mathfrak{M}$. To each $F \in [F]$ corresponds a mapping $\pi_1 F$ of U onto $\pi_1(X) \subset \Sigma$, and to each $\mathfrak{F} \in [\mathfrak{F}]$ corresponds a mapping $\pi_2 \mathfrak{F}$ of $\mathfrak{U}$ onto $\pi_2(\mathfrak{X}) \subset \Sigma$. We show that the set of these mappings defines a C^m-structure on Σ.

3. *Differential Transformation of the problem.*—We establish a fundamental lemma. Let $(x_1, \ldots, x_n)$ and $(y_1, \ldots, y_n)$ be Euclidean coordinates in E and $\mathfrak{E}$, respectively. Let I be the mapping of E onto $\mathfrak{E}$ under which $y_i = x_i$, $i = 1, \ldots, n$. We define a sense index $\sigma(\phi) = \pm 1$ in an obvious way, and say that ϕ is sense preserving if $\sigma(\phi) = 1$.

LEMMA 3.1 (*i*). *Given a sense preserving C^m-diff. ϕ of S_{n-1} or to $\mathfrak{M}_{n-1}$, $m > 0$, and an arbitrary point Q of S_{n-1}, there exists a C^m-diff. f of $\mathfrak{E}$ onto $\mathfrak{E}$ such that $f\phi$ is a sense preserving C^m-diff. of S_{n-1} onto $f(\mathfrak{M}_{n-1})$, and such that, for some neighborhood N of Q relative to S_{n-1}*

$$(f\phi)|N = I|N.$$

(ii). *There also exists a sense preserving C^m-diff. g of $\mathfrak{E}$ onto $\mathfrak{E}$, such that $g\phi$ is a sense preserving C^∞-diff. of S_{n-1} onto $g(\mathfrak{M}_{n-1})$.*

Let (L_1) and (L_2) be two classes of problems related to the Schoenflies problem. We say that the class (L_1) is *effectively* mapped into the class (L_2), if to each problem $L_1 \in (L_1)$ there corresponds at least one problem $L_2 \in (L_2)$ such that the existence of a solution of L_2 implies a solution of L_1. We shall identify (L_1) with the class of problems: given ϕ in Theorem 1.1, to find Λ_ϕ. In Section 4 we shall define a class of problems into which (L_1) can be effectively mapped.

4. *Problems of Type K.*—Let K be the n-cube in E,

$$(-1 \leqq x_i \leqq 1) \qquad (i = 1, \ldots, n)$$

and $\mathring{K}$ its interior. Set $\mathring{K}|(x_n = 0) = k$. The set $\mathring{K} - k$ is the union of two disjoint open subsets K' and K'' on which $x_n < 0$ and $x_n > 0$, respectively. Let G and B be compact subsets of $\mathring{K}$ such that $B \supset G$. Let ω be a C^∞-diff. of $\mathring{K} - G$ into $\mathring{K}$

which reduces to I on $\mathring{K} - B$. Let d be so small a positive constant that the subset H of $\mathring{K}$ on which $|x_n| > d$ includes G. Set

$$H' = H \cap K', \qquad H'' = H \cap K''; \qquad G' = G \cap K', \qquad G'' = G \cap K''.$$

Subsets of $\mathfrak{E}$: Setting $IK = \mathfrak{K}$ and $\omega(k) = \mathfrak{k}$, note that $\mathring{\mathfrak{K}} - \mathfrak{k}$ is the union of two disjoint open sets, $\mathfrak{K}'$ and $\mathfrak{K}''$, distinguished by the conditions,

$$I(K' - B) \subset \mathfrak{K}', \qquad I(K'' - B) \subset \mathfrak{K}''.$$

Introduce the subsets $\mathfrak{G} = \mathfrak{G}' \cup \mathfrak{G}''$, $\mathfrak{H} = \mathfrak{H}' \cup \mathfrak{H}''$ of $\mathfrak{K}$, where

$$\mathfrak{H}' = \mathfrak{K}' - \omega(K' - H'), \qquad \mathfrak{H}'' = \mathfrak{K}'' - \omega(K'' - H''),$$

$$\mathfrak{G}' = \mathfrak{H}' - \omega(H' - G'), \qquad \mathfrak{G}'' = \mathfrak{H}'' - \omega(H'' - G'').$$

A problem of type K is determined by the sets G and B, the mapping ω, and the constant d: it is to find a mapping λ_ω which satisfies the following lemma.

LEMMA 4.1. *There exists a* C_0^∞*-diff. of* H' *onto* $\mathfrak{H}'$ *such that for some compact subset* Ω *of* H', *with* $\Omega \supset G'$, $\lambda_\omega(p) = \omega(p)$ *for* $p \in H' - \Omega$.

LEMMA 4.2. *The class* (L_1) *of Schoenflies problems defined by Theorem 1.1 can be effectively mapped into the class of problems of type* K.

The Composite n-Manifolds X *and* X^*—Let R be a radial transformation of E onto E in which a point (x) has an image (y) such that $2y_1 - 16 = x_1 - 8$, $2y_r = x_r$, $r = 2, \ldots, n$. The point $P = (8, 0, \ldots, 0)$ in E is fixed under R. Let $\mathfrak{R}$ be similarly defined on $\mathfrak{E}$, with fixed point $\mathfrak{P}$. Let ω be extended in definition by setting

$$\omega R^r(\mathrm{p}) = \mathfrak{R}^r \omega(\mathrm{p}) \qquad (\mathrm{p} \in \mathring{K} - G).$$

Let $\boldsymbol{X}$ and $\boldsymbol{X}^*$ be composite sets, defined as is Σ in Section 2, canonically represented in the form,

$$\boldsymbol{X} = [M, \mathfrak{M}, \mu, W, \mathfrak{W}], \qquad \boldsymbol{X}^* = [M^*, \mathfrak{M}^*, \mu^*, W^*, \mathfrak{W}^*]$$

setting

$$\begin{cases} W = \bigcup_{r=0}^{\infty} R^r(H - G), & \mathfrak{W} = \bigcup_{r=0}^{\infty} \mathfrak{R}^r(\mathfrak{H} - \mathfrak{G}), \\ M = E - P - \bigcup_{r=0}^{\infty} R^r(G), & \mathfrak{M} = \bigcup_{r=0}^{\infty} \mathfrak{R}^r(\mathfrak{H}), \end{cases}$$

$$\begin{cases} W^* = H' - G', & \mathfrak{W}^* = \mathfrak{H}' - \mathfrak{G}', \\ M^* = E - P - G', & \mathfrak{M}^* = \mathfrak{H}', \end{cases}$$

and $\mu = \omega | W$, $\mu^* = \omega | W^*$. We show that $\boldsymbol{X}$ and $\boldsymbol{X}^*$ are n-manifolds with C^∞-structures as defined in Section 2.

The C^∞*-diff.* $\boldsymbol{s}$ *and* $\boldsymbol{t}$: Let π_1, π_2 and π_1^*, π_2^* be π-mappings associated, respectively, with $\boldsymbol{X}$ and $\boldsymbol{X}^*$, as in Section 2. We prove that there exists a C^∞-diff., $\boldsymbol{t}$ of $\boldsymbol{X}$ onto $\mathfrak{E} - \mathfrak{P}$, defined by the conditions,

$$(\boldsymbol{t}\,\pi_1)(p) = \omega(p) \qquad (r \geqq 0) \quad [p \in R^r(\mathring{K} - G)],$$
$$(\boldsymbol{t}\,\pi_2)(q) = q \qquad (r \geqq 0) \quad [q \in \mathfrak{R}^r(\mathfrak{H})],$$

and by the supplementary condition that $(\boldsymbol{t}\,\pi_1)(p) = I(p)$ for all $p \in M$ for which $(t\,\pi_1)(p)$ is not already defined. We further define a C^∞-diff. $\boldsymbol{s}$ of $\boldsymbol{X}$ onto $\boldsymbol{X}^*$, utilizing

a C^∞-diff. T of E onto E. The mapping T is characterized as follows. Let Int Z denote the smallest product of n subintervals of the respective coordinate axes of E which contains a bounded subset Z of E. Let $\boldsymbol{u}$ be the identity map of E. We define T so that

$$T|H'' = \boldsymbol{u}|H'', \qquad T|H'_{,} = R|H',$$
$$RT(K) \cap T(K) = 0, \qquad T(\mathring{K}) \subset \text{Int } [\mathring{K} \cup R\mathring{K}],$$

and so that under T the sign of the coordinate x_n remains invariant.

LEMMA 5.1. *The mapping* $\boldsymbol{k} = \boldsymbol{s}\,\boldsymbol{t}^{-1}\,\mathrm{I}$ *is a* C^∞*-diff. of* $E - P$ *onto* $\boldsymbol{X}^*$.

6. *Solution of a Problem of Type K.*—Let D be an n-interval in E, geometrically similar to H', containing P, and with the vertex (a) in common with H' $(a_i = -1;\ i = 1, \ldots, n)$. Let $D_1 \supset G'$ be a second n-interval, geometrically similar to H' and D, with vertex (a), and with $H' \supset \bar{D}_1$. Note that $D \supset D_1$. We define a C^∞-diff. $\boldsymbol{a}$ of D onto H', which leaves D_1 pointwise invariant. The open subsets

$$X = [M^* \cap D, \mathfrak{M}^*, \boldsymbol{X}^*], \qquad Y = [H' - G', \mathfrak{H}', \boldsymbol{X}^*],$$

of $\boldsymbol{X}^*$ are introduced, and proved to be μ^*-represented. The point $\boldsymbol{a}(P)$ is in $H' - G'$ and the point $Q = \pi_1^*[\boldsymbol{a}(P)]$ is in Y. With the aid of $\boldsymbol{a}$, a C^∞-diff. $\boldsymbol{D}$ of X onto $Y - Q$ is defined. The map

$$p \to (\pi_2^*)^{-1}[\boldsymbol{D}(\boldsymbol{k}[\boldsymbol{a}^{-1}(p)])] \qquad [p \in H' - \boldsymbol{a}(P)]$$

defines a C^∞-diff. of $H' - \boldsymbol{a}(P)$ into $\mathfrak{H}'$ and, if completed by mapping $\boldsymbol{a}(P) \to \omega[\boldsymbol{a}(P)]$ is a C_0^∞-diff. λ_ω of H' onto $\mathfrak{H}'$.

THEOREM 6.1. *Each problem of type K has a solution, thereby implying a solution of an arbitrary Schoenflies problem in Theorem* 1.1.

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TRANSFORMATION OF BIOCHEMICALLY DEFICIENT STRAINS OF BACILLUS SUBTILIS BY DEOXYRIBONUCLEATE

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INTRODUCTION

The experimental transfer of genetic characters by deoxyribonucleate (DNA) represents a powerful technique for the investigation of nucleic acid function at the molecular level.[1] The present study was initiated to obtain new microbial systems in which transformation of specific biochemical factors could be readily studied. By employing germinating spores of nutritionally deficient strains

of *Bacillus subtilis*, it was found that DNA isolated from a wild-type strain can transform these bacteria to nutritional independence. These new systems provide simple models for the study of the mechanism of genetic determinations and the biochemical control of enzyme synthesis.

METHODS

Strains of B. subtilis.—The strains of *B. subtilis* were obtained from Dr. Charles Yanofsky and originated from a collection of Burkholder and Giles[2] at Yale University.

Wild-type *B. subtilis* 23[3] was employed for the isolation of transforming DNA. The following strains of nutritionally deficient *B. subtilis* were used as recipients for the transformation studies:

Strain No.	Growth Requirement
168	L-Tryptophane, or indole
166	L-Tryptophane, indole, or anthranilic acid
122	Nicotinic acid

Preparation of Spores.—Potato agar medium used for the preparation of the spores of the recipient strains was prepared as follows: 200 gm. of peeled, chipped potatoes were boiled in 1 liter of tap water, filtered through cotton, and, after the addition of 5 mg. of manganese sulfate, the pH was adjusted to 6.8. The volume was then adjusted to 1 liter with tap water, 15 gm. of Difco agar was added, and the medium was autoclaved at 15 lb. pressure for 15 minutes.

Cultures (from nutrient agar slants) were heavily streaked on potato agar slants. The latter were incubated at 37° C. for 7 days and then refrigerated at 4° C. for at least 7 days. Spores were also prepared by growth on Difco Tryptose-Blood Agar Base slants for 3 days at 37° C.

Isolation and Purification of DNA.—DNA was isolated from cells of *B. subtilis* 23 grown with shaking at 37° C. in Difco Penassay broth for 18 hours. The packed cell sediment (from Sharples centrifugation) was disintegrated in a modified Hughes Press[4, 5] which had been cooled to −20° C. The crushed frozen paste was suspended in cold 0.1 *M* sodium chloride solution containing 0.05 *M* sodium citrate (approximately 10 ml. for each gram of wet cells) by homogenizing with a Ten Broeck glass homogenizer. The resulting viscous suspension was centrifuged at 30,000 × *g.* for 20 minutes at 0° C., and the supernatant was discarded. The sediment was then washed with cold 0.1 *M* sodium chloride and 0.05 *M* sodium citrate solution, and then was suspended in cold 2 *M* sodium chloride solution. After agitation with a magnetic stirrer at 4° C. for 30 minutes, the suspension was centrifuged at 20,000 × *g.* for 20 minutes. The supernatant liquid was slowly added to 2.5 volumes of cold 95 per cent ethanol, and the fibrous precipitate was rapidly removed with hooked stirring rods to avoid contamination by the subsequent fine precipitate. The fibers were dried by suction on a filter-paper pad on a Buchner funnel.

The dried fibers were suspended with the aid of a glass Ten Broeck homogenizer in cold 2 *M* sodium chloride solution. The following procedures were employed to remove the protein still associated with the DNA at this stage: After 18 hours at 4° C., sodium lauryl sulfate was added to a concentration of 0.5 per cent, and after 10 minutes at room temperature the suspension was chilled in an ice bath and

centrifuged at 4° C. at 2,500 × *g*. for 10 minutes. The supernatant fluid was then shaken with an equal volume of a chloroform-octanol (5:1) mixture for 15 minutes at room temperature and centrifuged at 2,500 × *g*. for 5 minutes. The aqueous layer was carefully aspirated from the whitish, gelatinous interface. The extractions with chloroform-octanol were repeated until no gelatinous interface could be observed, which usually required seven extractions.

The DNA was then precipitated as fibers from cold 80 per cent ethanol, dried on sterile filter paper on a Buchner funnel under suction, and restored in sterile 2 *M* sodium chloride solution. Most of the transformation studies reported in this communication were performed with the preparations purified in this manner. Sterility of the DNA preparations was essential in the transformation studies, and it was found that this was insured by the sodium lauryl sulfate and chloroform-octanol treatments.

For estimating components of the DNA preparations, the following procedures were employed. Protein determinations were made by the Folin-Phenol method of Lowry,[6, 7] using crystalline bovine albumin as standard. DNA was estimated by the diphenylamine reaction as modified by Burton,[8] using a thymus DNA preparation as standard. Ribonucleic acid was estimated by the orcinol method,[9] using *d*-ribose as standard.

Assay Procedure.—A sterile glucose minimal medium of the following composition was employed in the transformation studies:

	Per Cent
Ammonium sulfate	0.2
Dipotassium phosphate	1.4
Monopotassium phosphate	0.6
Sodium citrate ($2H_2O$)	0.1
Magnesium sulfate ($7H_2O$)	0.02
Glucose	0.5

The glucose was sterilized separately and added aseptically.

Spores of the auxotrophs from potato agar slants were suspended in glucose-minimal medium at concentrations of approximately 10^8 spores/ml, as determined by viable counting on Difco Heart Infusion Agar plates and by direct microscopic examination. The suspensions were heated at 60° C. for 5 minutes (to activate the spores for germination and to inactivate vegetative cells) and then were diluted tenfold in glucose-minimal supplemented with 0.01 per cent Difco yeast extract (2.5 ml/tube). In transformation experiments wild-type donor DNA was then added to this medium. Controls were run without DNA as well as with DNA treated with deoxyribonuclease[10] (DNAase; 2–20 μg/ml + 0.005 *M* magnesium sulfate for 30 minutes at 37° C. prior to addition to the glucose minimal). The tubes were incubated at 37° C. on a reciprocal shaker. At suitable intervals, 2–20 μg. DNAase and magnesium sulfate to give a concentration of 0.005 *M* were added and 0.1-ml. aliquots plated on glucose-minimal agar for detection of nutritionally independent transformants. In instances where additional substances were present which could support the growth of the auxotroph, the suspensions were washed with sterile 0.5 per cent sodium chloride solution before plating.

RESULTS

Demonstration of DNA Transforming Activity.—When suspensions of spores of the indole-requiring *B. subtilis* 168 were incubated with sterile preparations of

DNA isolated from *B. subtilis* 23, transformation to organisms no longer requiring indole for growth was observed. The transformation to indole independence is completely prevented by treatment of the donor DNA with 2 μg/ml or more of crystalline DNAase in the presence of Mg ions. Spontaneous reversion to non-requirement has never been observed with this strain, (i.e., in the absence of DNA from a "wild-type" donor). DNA isolated from strain 168 or from a number of *Escherichia coli* strains is inactive. As little as 2×10^{-3} μg. DNA per ml. (isolated from strain 23) produces transformation, and the number of transformants is a function of the quantity of DNA employed.

The DNA isolated from transformed cells derived from strain 168 was found to be more effective in transforming strain 168 to indole independence than the DNA derived from strain 23. Thus in the former as little as 2×10^{-4} μg. DNA per ml. had transforming activity. This represents an increase in specific activity of at least tenfold over that with DNA derived from strain 23.

Table 1 presents data from a typical experiment in which a suspension of 5×10^7

TABLE 1*

TRANSFORMATION OF *B. subtilis* 168 (INDOLE-) BY DNA FROM WILD TYPE

DNA (μg/ml)	Transformants (= Colonies on Minimal Agar per ml.) ($\times 10^2$)
20	7,000
2	4,700
0.2	2,800
0.02	480
0.002	26
0.001	12
0.0005	5.9
0.00025	2.7
0.00012	1.0
0	0
20 (+ DNAase 2 μg. + 0.005 *M* Mg)	0

* *Experimental Conditions.*—*B. subtilis* 168, 3-day growth on Tryptose–Blood Agar base suspended with shaking 4 hours at 37° C. in glucose-minimal medium containing 0.1 per cent Bacto yeast extract. Then centrifuged and resuspended in glucose-minimal medium containing 0.01 per cent yeast extract and DNA (DNA isolated from wild-type *B. subtilis* strain, produced by transformation of strain 168 with DNA from strain 23, deproteinized by chloroform-octanol extraction; RNA was not removed from this preparation) at various levels. The latter incubation was at 37° C. for 1 hour with shaking. 20 μg. of DNAase was added, and the Mg^{++} concentration was brought to 0.005 *M*. Aliquots were plated on the minimal-glucose agar medium. Colonies were counted after 24 hours. Total number of cells as determined by plating on complete medium = 5×10^7/ml.

cells per ml. of strain 168 were incubated with graded doses of DNA isolated from a wild-type strain.

Similar results have been obtained with recipient germinating spores of strain 166, which requires anthranilic acid, indole, or tryptophane for growth, and with those of strain 122 which requires nicotinic acid.

Requirements for Transformation.—Although transformation of strain 168 could be obtained in a completely defined medium (the glucose-minimal medium), the activity of the DNA was greatly enhanced by the addition of 0.01 per cent Bacto yeast extract (Difco) (Table 2). The active component(s) in yeast extract has not been identified as yet. The yeast extract supplement could not be replaced by any of the known vitamins or ribonucleotides found in yeast.

Bovine serum albumin, known to be required in *Pneumococcus* transformations,[11] is inhibitory, even in the presence of yeast extract.

TABLE 2

STIMULATING EFFECT OF YEAST EXTRACT ON TRANSFORMATION

Addition to Glucose Minimal Medium	Minimal Conc. of *B. subtilis* 23 DNA Required for Transformation of Strain 168 (18 hr., 37° C.)
None	2 μg/ml
+ 0.01 per cent yeast extract (Difco)	0.002 μg/ml
+ 0.01 per cent yeast extract + 0.04 per cent bovine serum albumin	0.2 μg/ml
+ 0.5 per cent casein hydrolyzate	2 μg/ml

Purification of DNA from B. Subtilis 23.—Analysis of the isolated fractions for DNA, protein, and RNA revealed that a combination of sodium lauryl sulfate precipitation and chloroform-octanol extractions was required for removal of protein to approximately 1 per cent or less of the DNA (Table 3).

TABLE 3

FRACTIONATION OF *B. subtilis* 23 DNA

Fraction	DNA (μg/ml)	Protein (μg/ml)	RNA (μg/ml)
I. First alcohol precipitate restored in 2 *M* NaCl	700	1,500	. .
II. Supernatant fluid from sodium lauryl sulfate treatment	600	400	300
III. Second alcohol precipitate restored in 2 *M* NaCl (after 7 chloroform-octanol extractions)	600	<10	320
IV. Fraction III treated with RNAase (50 μg/ml), alcohol precipitated, and restored in 2 *M* NaCl solution	600	<10	<10

It can be seen from Table 4 that protein removal increased the activity 100–1,000 fold. This is somewhat surprising, since protein removal would presumably make the DNA more susceptible to cellular DNAase. On the other hand, protein removal may produce a smaller molecular species more readily able to penetrate the recipient cells.

Removal of RNA could be achieved by ribonuclease treatment (50 μg/ml of crystalline Worthington ribonuclease, 30 minutes, 37° C.) followed by alcohol

TABLE 4

TRANSFORMING ACTIVITIES OF *B. subtilis* 23 DNA FRACTIONS

Fraction	Minimal Conc. of DNA Required for Transformation of Strain 168 (μg/ml) (18 hr., 37° C.)
I. First alcohol precipitate restored in 2 *M* NaCl	0.2–2.0
III. Second alcohol precipitate restored in 2 *M* NaCl (after 7 chloroform-octanol extractions)	0.002
IV. Fraction III treated with RNAase, precipitated with alcohol and restored in 2 *M* NaCl	0.02
IV. + RNA fraction*	0.004

* The RNA was obtained as follows: Fraction III (5 ml. containing 600 μg/ml DNA) was treated with 50 μg/ml of crystalline DNAase and 0.005 *M* $MgSO_4$ for 30 minutes at 37° C., followed by the addition of 0.01 *M* sodium citrate. Cold ethanol was added to give a concentration of 70 per cent. A fine precipitate formed, which was collected on filter paper and was restored to the original volume with 2 *M* NaCl solution. This preparation was found to contain all the RNA originally present as determined by the orcinol reaction, but no DNA. The amount of RNA added to Fraction IV was equivalent to the original quantity present.

precipitation. Norite treatment[12] did not remove this RNA fraction, which may be closely associated with the DNA.

The presence of an RNA component appears to have potentiating activity. Its complete removal, i.e., after alcohol precipitation following ribonuclease treatment, lowers the biological activity of the DNA preparation approximately tenfold. This loss in activity can be partially restored by the addition of the RNA fraction (see Table 4).

Stability of the DNA.—The transforming activity of the purified DNA appears to be very stable in 2 *M* sodium chloride solution at 4° C. Reproducible activity has been observed over a period of months. Approximately 99 per cent of the transforming activity was destroyed by storage in dilute sodium chloride (0.1 *M*) at 4° C. for 2 days.

Transforming activity of purified DNA in 2 *M* NaCl is reduced to the extent of 99.9 per cent of the original by slow freezing at −20° C. and subsequent thawing at 37° C. On the other hand, marked stability to heat has been observed. When heated at 70° C. for 30 minutes, the minimal quantity of DNA capable of effecting transformation remained unchanged, but the number of transformants produced with any given quantity of DNA was reduced.

DISCUSSION

The present studies have demonstrated the transformation of biochemically deficient strains of *Bacillus subtilis* to nutritional independence by deoxyribonuclease-sensitive deoxyribonucleate isolated from a "wild-type" strain. Spores of the auxotrophs when allowed to germinate in a minimal medium containing glucose and 0.01 per cent of an autoclaved yeast extract could be readily transformed with as little as 2×10^{-4} μg DNA/ml. Little or no transformation occurred with the auxotrophic recipient vegetative cells actively multiplying in nutrient medium. The results obtained suggest that germinating spores may be particularly susceptible to transformation with DNA, possibly at a particular growth stage in which the cell wall is still incomplete. The DNA may be diverted in mature vegetative cells by combination with cell-wall components, such as the free amino groups of hexoseamines, diaminopimelic acid, etc. The observed presence of a DNA slime layer surrounding the cell walls of a number of bacterial species[13–14] supports this view.

The possibility that cell wall removal could allow the transfer of DNA has been explored by a number of workers. Disrupted T_2 virus which cannot infect intact cells will do so if the cell wall components are removed as in protoplasts.[15–17] Chargaff *et al.*[18] have reported the transformation of a lysine-requiring mutant of *Escherichia coli*, strain W, by the use of protoplasts of this organism and a fraction containing DNA from the wild-type strain. The small number of transformants observed may be due to poor survival as a result of the osmotic fragility of protoplasts and the complexities of cell-wall resynthesis. Lederberg and St. Clair,[19] however, have also attempted transformation of protoplasts of a number of mutants, but without success.

The activity of donor DNA appears to be increased by a ribonucleic acid (RNA) moiety closely associated with the DNA. One possible mechanism by which the RNA exerts this effect may be its activity as a specific inhibitor of deoxyribonuclease.[13a, 20, 21]

Of considerable interest is the large increase in activity of transforming DNA

achieved by protein removal. The protein remaining after detergent treatment is apparently closely associated with the DNA and may prevent easy entrance into the recipient cells by maintaining a large molecular complex.

SUMMARY

Three auxotrophic mutants of *B. subtilis* have been transformed to nutritional independence with deoxyribonucleate (DNA) isolated from a wild-type *B. subtilis*, strain 23. As little as 2×10^{-4} μg. of DNA effects transformation of heat-treated spores allowed to germinate in a glucose-minimal medium. The number of transformants produced is directly related to the quantity of DNA added and transformation is prevented by treatment with deoxyribonuclease. The specific activity of the donor DNA is increased by removal of protein, and it also appears that a ribonucleate fraction, closely associated with donor DNA, enhances transformation activity. An unidentified factor in yeast extract also appears to be required for efficient transformation. The effective DNA is relatively labile to freezing and storage in solutions of low ionic strength but is relatively stable in 2 *M* NaCl solutions.

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*THE APPLICATION OF THE FICK PRINCIPLE TO THE MEASUREMENT OF PULMONARY BLOOD FLOW**

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The Fick[1] method is fundamentally sound and under ideal conditions should exactly measure the pulmonary flow. But under experimental conditions its accuracy is less certain, and an assessment of this accuracy is the purpose of this report.

THEORETICAL BACKGROUND

The Fick method is based on the principle that the volume of oxygen entering the pulmonary capillaries equals the volume of oxygen gained by the flowing blood. When these volumes are expressed in terms of time, the following equation is obtained:

$$V_{O_2}(t) = Q_0(t)C_0(t) - Q_i(t)C_i(t), \tag{1}$$

where $V_{O_2}(t)$ = instantaneous rate of oxygen uptake by the capillaries, $Q_0(t)$ = instantaneous rate of blood flow from the capillaries, $Q_i(t)$ = instantaneous rate of blood flow into the capillaries, $C_0(t)$ = instantaneous concentration of oxygen in the blood flowing from the capillaries, and $C_i(t)$ = instantaneous concentration of oxygen in the blood flowing into the capillaries.

An important feature of this equation is that it can be solved for a single value of flow only if $Q_0(t) = Q_i(t)$. Hence, when

$$Q_0(t) = Q_i(t) = Q(t),$$

then

$$V_{O_2}(t) = Q(t)[C_0(t) - C_i(t)]$$

and

$$Q(t) = \frac{V_{O_2}(t)}{[C_0(t) - C_i(t)]}. \tag{2}$$

By integrating this equation, the average flow, Q_{av}, during any finite period, T, can be obtained thus:

$$Q_{av} = \frac{1}{T}\int_0^T Q(t)dt = \frac{1}{T}\int_0^T \left\{\frac{V_{O2}(t)}{[C_0(t) - C_i(t)]}\right\}dt. \tag{3}$$

For the derivation of equations (2) and (3) it is only necessary that $Q_0(t)$ and $Q_i(t)$ be equal. But for their application it is also necessary that the following conditions should be met:

1. The volume of blood in the capillaries should be constant, and the entering oxygen should be instantaneously and homogeneously mixed with the blood.
2. All particles of blood should traverse the capillary bed in zero time.
3. There should be means for continuously measuring (*a*) the rate at which oxygen enters the capillaries and (*b*) the concentrations of oxygen in the blood entering and leaving the capillaries.

Under these conditions, the instantaneous flow could be followed by continuously solving equation (2), while the average flow could be calculated from equation (3).

POTENTIAL SOURCES OF ERROR

In practice, each of the specified conditions is compromised. The principal difficulty stems from the fact that the variables must be measured at sites remote from the capillary bed. The time delays thereby introduced vitiate the calculation of instantaneous flow and render the calculation of average flow complex.

This complexity is caused primarily by the following factors: (1) the necessity for measuring the oxygen uptake at the mouth, (2) the impossibility of measuring the variables instantaneously, and (3) the uncertainty of the relationship between $Q_0(t)$ and $Q_i(t)$ at the points where blood is sampled. The importance of these factors will be considered next.

Necessity for Measuring Oxygen Uptake at the Mouth.—The rate at which oxygen enters the capillaries must be estimated from the rate of oxygen uptake at the mouth, an undesirable procedure since it is doubtful that these two variables are instantaneously equal. Even the equality of the average values is uncertain, since the lungs constitute a reservoir in which oxygen can be temporarily stored or lost. If storage or loss occurs during the period of measurement, the uptake will no longer equal the rate of entry into the capillaries, and an error will be introduced into the calculated flow. In the basal subject, this error is probably unimportant, particularly if the period of measurement is much longer than the time of a single breath. But the error may become important in any experiment in which either the mean lung volume or the composition of the alveolar air is altered. In such an experiment, it is essential that the uptake be measured only after these variables have reached a new steady state.

Impossibility of Measuring the Variables Instantaneously.—The blood concentrations of oxygen are necessarily determined from samples collected over a finite period. Visscher and Johnson[2] have called attention to an error which may thus be introduced. This error is best explained by using an example.

Let us assume that during a finite interval the average rate of oxygen absorption is measured at the capillary bed. Let us also assume that blood is sampled from the ends of the capillaries by withdrawing the plungers of two sampling syringes at the same constant rate. By analyzing these specimens, the data necessary for the usual Fick calculation are obtained. This calculation is expressed in general terms thus:

$$Q_{Fick} = \frac{1/T\int_0^T V_{O_2}(t)dt}{1/T\int_0^T C_0(t)dt - 1/T\int_0^T C_i(t)dt} = \frac{\int_0^T V_{O_2}(t)dt}{\int_0^T [C_0(t) - C_i(t)]dt}. \quad (4)$$

Although equation (4) presumably measures the average flow, it is obviously different from equation (3). Hence the question arises as to whether Q_{Fick} and Q_{av} are equal. This question can be answered by equating (4) and (3) and determining the conditions necessary for identity. Thus

$$\frac{\int_0^T V_{O_2}(t)dt}{\int_0^T [C_0(t) - C_i(t)]dt} = \frac{1}{T}\int_0^T \left\{\frac{V_{O_2}(t)}{[C_0(t) - C_i(t)]}\right\} dt. \quad (5)$$

The expressions are obviously dissimilar, but it can be shown that an identity is established when either $\{V_{O2}(t)/[C_0(t) - C_i(t)\}$ or $[C_0(t) - C_i(t)]$ is constant.

Stow[3] approached the sampling problem in a different way by directing attention to the fact that when blood is collected at a constant rate from a vessel in which the flow is varying, the concentration of oxygen in the syringe will not always equal the concentration of oxygen in the volume of blood passing the sampling site. He illustrated the point by writing expressions for the two concentrations:

$$C_s = \frac{1/T\int_0^T FC(t)dt}{1/T\int_0^T Fdt} = \frac{1}{T}\int_0^T C(t)dt \quad (6)$$

and

$$C_v = \frac{1/T\int_0^T K_s Q(t)C(t)dt}{1/T\int_0^T K_s Q(t)dt} = \frac{\int_0^T Q(t)C(t)dt}{\int_0^T Q(t)dt}, \quad (7)$$

where C_s = concentration of oxygen in the blood in the syringe; C_v = concentration of oxygen in the volume of blood passing the sampling site; F = constant = rate at which blood is sampled; and K_s = constant = factor relating the rate of sampling to the rate of intravascular flow. Each of these relations can be expanded into an expression for the arteriovenous oxygen difference $(A - V)$. Hence

$$(A - V)_s = \frac{1}{T}\int_0^T [C_0(t) - C_i(t)]dt, \quad (8)$$

$$(A - V)_v = \frac{\int_0^T Q(t)[C_0(t) - C_i(t)]dt}{\int_0^T Q(t)dt}. \quad (9)$$

Again, as indicated for equations (3) and (4), it can be shown that these expressions will be equal and the error obviated whenever the flow is constant or the arteriovenous oxygen difference is fixed.

These considerations bring an important question into focus. If the measured average oxygen uptake were reliable and if it were possible to obtain blood samples in a manner whereby the collected volumes were proportional to the intravascular flow, could the average pulmonary flow be calculated, regardless of the variation of $V_{O2}(t)$, $C_0(t)$, and $C_i(t)$? The question can be answered by determining whether the flow (Q_{Fick}) calculated under these conditions would equal equation (3). Thus

$$Q_{Fick} = \frac{1/T\int_0^T V_{O_2}(t)dt}{\left[\frac{1/T\int_0^T K_s Q(t)C_0(t)dt}{1/T\int_0^T K_s Q(t)dt}\right] - \left[\frac{1/T\int_0^T K_s Q(t)C_i(t)dt}{1/T\int_0^T K_s Q(t)dt}\right]}. \quad (10)$$

When equation (10) is simplified and set equal to equation (3), the following expression is obtained:

$$\frac{1/T\int_0^T V_{O_2}(t)dt}{\left\{\frac{\int_0^T Q(t)[C_0(t) - C_i(t)]dt}{\int_0^T Q(t)dt}\right\}} = \frac{1}{T}\int_0^T \left\{\frac{V_{O_2}(t)}{[C_0(t) - C_i(t)]}\right\} dt.$$

But, since

$$Q(t) = \frac{V_{O_2}(t)}{[C_0(t) - C_i(t)]}$$

then

$$\frac{1/T\int_0^T V_{O_2}(t)dt}{\frac{\int_0^T \left\{\frac{V_{O_2}(t)}{[C_0(t) - C_i(t)]}\right\}[C_0(t) - C_i(t)]dt}{\int_0^T \left\{\frac{V_{O_2}(t)}{[C_0(t) - C_i(t)]}\right\} dt}} = \frac{1}{T}\int_0^T \left\{\frac{V_{O_2}(t)}{[C_0(t) - C_i(t)]}\right\} dt,$$

whence

$$\frac{1}{T}\int_0^T \left\{\frac{V_{O_2}(t)}{[C_0(t) - C_i(t)]}\right\} dt = \frac{1}{T}\int_0^T \left\{\frac{V_{O_2}(t)}{[C_0(t) - C_i(t)]}\right\} dt. \qquad (11)$$

Thus, since equation (11) indicates that equations (10) and (3) are equal, the average pulmonary flow could be calculated regardless of variations in oxygen uptake, blood oxygen concentrations, or flow, provided, first, that the average oxygen uptake could be reliably measured and, second, that a constant relationship between the volume of blood collected and the rate of intravascular flow could be maintained.

The Uncertainty of the Relationship between $Q_0(t)$ and $Q_i(t)$ at the Points Where Blood Is Sampled.—In the foregoing discussion, equation (3) has been considered to be a reliable expression for calculating average flow. But it must be remembered that this expression has as its basis the assumption that the instantaneous rates of inflow and outflow are equal. In view of the elastic nature of the vessels and the pulsatile character of the flow, the validity of this assumption is uncertain. It is probably more realistic to assume that $Q_0(t)$ and $Q_i(t)$ are not equal but that their averages over a prolonged period are the same. Hence it is worthwhile to consider whether in this circumstance the Fick principle can be used at all.

The problem can be approached by rewriting equation (1) in terms of the finite interval, T:

$$\frac{1}{T}\int_0^T V_{O_2}(t)dt = \frac{1}{T}\int_0^T Q_0(t)C_0(t)dt - \frac{1}{T}\int_0^T Q_i(t)C_i(t)dt. \qquad (12)$$

Further, the assumption that the average inflow and outflow are equal can be expressed as follows:

$$\frac{1}{T}\int_0^T Q_0(t)dt = \frac{1}{T}\int_0^T Q_i(t)dt = Q_{av}. \qquad (13)$$

An examination of these two equations makes evident an important fact. Equation (12) is readily soluble only when $C_0(t)$ and $C_i(t)$ are constant. In this circumstance,

$$\frac{1}{T}\int_0^T V_{O_2}(t)dt = \frac{C_0}{T}\int_0^T Q_0'(t)dt - \frac{C_i}{T}\int_0^T Q_i(t)dt$$

and

$$\frac{1}{T}\int_0^T V_{O_2}(t)dt = (C_0 - C_i)Q_{av},$$

whence

$$Q_{av} = \frac{1/T\int_0^T V_{O_2}(t)dt}{C_0 - C_i}, \tag{14}$$

where $C_0(t) = C_0 =$ constant and $C_i(t) = C_i =$ constant.

By comparing equation (14) with the usual Fick calculation, it is apparent that the two are identical when $C_0(t) = C_0$ and $C_i(t) = C_i$. Therefore, when the instantaneous rates of inflow and outflow are not equal but their averages are the same, the reliability of the Fick method is directly related to the constancy of the oxygen concentrations in the arterial and mixed venous blood.

METHODS FOR MINIMIZING THE POTENTIAL ERRORS

The problem faced in evaluating the Fick method is the same as that encountered in evaluating any method for measuring flow. This problem stems from the fact that, since flow cannot be measured directly in the intact animal, an alternative approach to the problem of evaluation must be employed. This approach consists of identifying the sources of error and assessing the extent to which each error can be minimized.

From the analysis in the preceding section, it is evident that the accuracy of the Fick method depends chiefly on these conditions: (1) the exactness with which the oxygen uptake measured at the mouth reflects the rate at which oxygen enters the capillary bed; (2) the constancy of the concentrations of oxygen in the arterial and mixed venous blood; and (3) the extent to which the average outputs of the right and left ventricles determined over a finite period are the same. In addition, the accuracy also depends on the precision with which the samples of blood and gas are analyzed.

To insure that these conditions are approximated, it has been previously suggested that the Fick method be applied only in the steady-state.[4, 5] This is particularly important in experiments where either the subject exercises or his breathing mixture is changed. In both circumstances the composition of the alveolar air is altered, and during the period of readjustment the measured oxygen uptake is not a reliable index of the oxygen delivered to the blood. It is essential, therefore, that the gas exchange reach a steady level before the measurement is made.

A related error may be introduced if the oxygen uptake measured at the mouth includes the oxygen involved in metabolizing lactic acid or other substances present in the blood traversing the vessels of the lungs. However, simultaneous measure-

ments of lactic acid in arterial and mixed venous blood samples collected under a variety of conditions have shown that this error can be dismissed.[6]

The constancy of the oxygen concentration in the mixed venous blood has been studied by Wood, Bowers, Shepherd, and Fox.[7] They found that the variations in oxygen concentration in the blood in the pulmonary arteries of basal subjects were small. During exercise, however, the variations were larger. These data are similar to those obtained in our laboratory by using a less elegant method to study the variations in oxygen concentration in brachial arterial blood.[8] The data suggest that, although the concentration of oxygen fluctuates, the fluctuations are small compared to a fairly constant mean.

The equality of the average outputs of the two ventricles cannot be measured, but there is no evidence of a significant difference over a prolonged period of time. Here again, however, numerous stimuli may alter the distribution of the total blood volume and can presumably change the volume of blood in the lungs. But it seems improbable that under any but extreme conditions a large enough change would occur during the period of measurement greatly to affect the calculated flow.

It is easier to evaluate the accuracy of the analyses of blood and gas samples. These analyses may introduce a maximum error of about 15 per cent. Thus it might be expected that the reproducibility of the measurement would be impaired by this factor, but in two early series of experiments from this laboratory two successive measurements agreed within 15 per cent in 98 of 100 basal subjects.[9, 10]

Finally, the Fick method has been criticized because during acute hypoxia it has been found to yield a value which is much smaller than that measured with dye.[11] But the measurements were made after only 5 minutes of severe hypoxia—too soon, undoubtedly, for the establishment of a new steady state. In general, evidence of such unsteadiness is provided, at least in normal individuals, by (*a*) a difference between the oxygen uptakes and respiratory quotients measured in the eupoxic and hypoxic states, and (*b*) by a lack of constancy of the arteriovenous oxygen difference during hypoxia.

We repeated the experiments under more nearly ideal conditions in 18 patients who were believed to have normal hearts and lungs. Each patient first breathed 21 per cent oxygen in nitrogen and then either 12 or 14 per cent oxygen in nitrogen. With the 21 per cent mixture, all except one set of Fick and dye measurements agreed within 25 per cent. After 20 minutes of hypoxia, the measurements were repeated and, again with a single exception, agreed within these limits.

Whereas it is reassuring to know that the Fick and dye-dilution methods give comparable results in a variety of circumstances, this does not establish the accuracy of either. The pitfalls in the Fick method have been mentioned. The dye-dilution technique also has potential sources of error, and these are equally difficult to assess.

EFFECT OF ANASTOMOSES BETWEEN PULMONARY AND BRONCHIAL VESSELS ON CALCULATED VALUE OF FLOW

The foregoing discussion emphasized the fact either that $Q_0(t)$ and $Q_i(t)$ must be equal or that their averages must be the same. We should like now to turn to a situation where these conditions are not realized. Such a situation can arise when blood from the bronchial vessels enters the pulmonary circulation via anastomoses within the lungs.

To investigate this problem we developed a method whereby the outputs of the right and left ventricles could be separately estimated. In brief, the method entailed injecting dye into the right atrium while inscribing simultaneous dilution curves from the pulmonary and brachial arteries. It was presumed that the former curve could be used for calculating the output of the right ventricle, and the latter for calculating the output of the left. In 22 experiments performed on 11 patients free of cardiopulmonary disease, the two values agreed within 15 per cent. The average difference for the entire group was zero.

With certain cardiopulmonary disorders, large anastomoses between the bronchial and pulmonary vessels are said to occur. These disorders include bronchiectasis and any situation in which a major branch of the pulmonary artery is occluded. To investigate the possibility that blood entered the pulmonary vessels through these anastomoses, we inscribed from one to three sets of simultaneous dilution curves in each of 12 patients with bronchiectasis and in 1 patient whose left main pulmonary artery had been ligated on the occasion of a left lower-lobe lobectomy. Although in the patients with bronchiectasis a difference between the flows from the pulmonary and brachial arteries was not a consistent finding, in some of the patients the discrepancy was striking. On the average, the brachial arterial flow exceeded that in the pulmonary artery by 10 per cent. In the patient with the ligated pulmonary artery, the output of the left ventricle was 20 per cent larger than the pulmonary arterial flow.

We believe that these differences are best explained by the presence of anastomoses. With such anastomoses, the outputs of the two ventricles would be unequal, and reference to either "the pulmonary flow" or "the cardiac output" would be meaningless. Thus the question arises as to whether the Fick method can be applied in this situation. The theoretical aspects of the problem can be examined by using the following hypothetical case.

Let us assume that anastomoses between the bronchial and pulmonary vessels carry blood with an oxygen concentration of $C_b(t)$ into the pulmonary circulation at the rate $Q_b(t)$. Let us also assume that $Q_{0av} = Q_{iav} + Q_{bav}$, and that the oxygen concentrations in the different streams are constant. These last two assumptions are the same as those made in analyzing the Fick equation in the first part of this report. And, lastly, let us assume that the recirculated blood will be distributed among the pulmonary vascular pathways as is the blood from the pulmonary artery. On these premises, equation (1) can be rewritten as follows:

$$\frac{1}{T}\int_0^T V_{O_2}(t)dt = \frac{1}{T}\int_0^T Q_0(t)C_0(t)dt - \frac{1}{T}\int_0^T Q_i(t)C_i(t)dt - \frac{1}{T}\int_0^T Q_b(t)C_b(t)dt.$$

But, when the blood concentrations of oxygen are constant, then

$$\frac{1}{T}\int_0^T V_{O_2}(t)dt = \frac{C_0}{T}\int_0^T Q_0(t)dt - \frac{C_i}{T}\int_0^T Q_i(t)dt - \frac{C_b}{T}\int Q_b(t)dt, \qquad (15)$$

where $C_0(t) = C_0 = \text{constant}$, $C_i(t) = C_i = \text{constant}$, and $C_b(t) = C_b = \text{constant}$. Further, on the assumption that $Q_{0av} = Q_{iav} + Q_{bav}$,

$$\frac{1}{T}\int_0^T Q_0(t)dt = \frac{T}{1}\int_0^T Q_i(t)dt + \frac{1}{T}\int_0^T Q_b(t)dt, \qquad (16)$$

and, substituting equation (16) in equation (15),

$$\frac{1}{T}\int_0^T V_{O_2}(t)dt = C_a\left[\frac{1}{T}\int_0^T Q_t(t)dt + \frac{1}{T}\int_0^T Q_b(t)dt\right] - C_t\left[\frac{1}{T}\int_0^T Q_t(t)dt\right] - C_b\left[\frac{1}{T}\int_0^T Q_b(t)dt\right] \quad (17)$$

from which

$$\frac{1}{T}\int_0^T V_{O_2}(t)dt = C_aQ_{tav} + C_aQ_{bav} - C_tQ_{tav} - C_bQ_{bav}.$$

When equation (17) is rewritten in familiar symbols and when both members are divided by $(C_a - C_{\bar{v}})$, the expression shown below is obtained:

$$\left[\frac{1}{C_a - C_{\bar{v}}}\right]\left\{\frac{1}{T}\int_0^T V_{O_2}(t)dt\right\} = \left[\frac{1}{C_a - C_{\bar{v}}}\right]\left\{C_aQ_{\mathrm{RV}_{av}} + C_aQ_{bav} - C_{\bar{v}}Q_{\mathrm{RV}_{av}} - C_bQ_{b_{av}}\right\}$$

or

$$\frac{1/T\int_0^T V_{O_2}(t)dt}{C_a - C_{\bar{v}}} = Q_{\mathrm{RV}} + \left[\frac{C_a - C_b}{C_a - C_{\bar{v}}}\right]Q_{b_{av}}, \quad (18)$$

where C_a = concentration of oxygen in the arterial blood, $C_{\bar{v}}$ = concentration of oxygen in the mixed venous blood, C_b = concentration of oxygen in the bronchial blood entering the pulmonary vessels, $Q_{\mathrm{RV}_{av}}$ = average rate of flow from the right ventricle, and Q_{bav} = average rate of flow through the anastomoses.

But the left-hand member of equation (21) is the usual Fick calculation when the blood concentrations of oxygen are constant. Hence

$$Q_{Fick} = Q_{\mathrm{RV}_{av}} + \left[\frac{C_a - C_b}{C_a - C_{\bar{v}}}\right]Q_{b_{av}}. \quad (19)$$

Inspection of equation (22) indicates that, under the specified conditions, the Fick measurement will include the flow from the bronchial vessels only if $C_b = C_{\bar{v}}$. If $C_b < C_{\bar{v}}$, Q_{Fick} will be larger than the output of the left ventricle, and if $C_b > C_{\bar{v}}$, Q_{Fick} will lie between the right and left ventricular flows. Finally, if $C_b = C_a$, the Fick will not measure the flow through the anastomoses at all.

Fick measurements were also made in the subjects previously mentioned. The data indicated that in the normal subjects the Fick agreed within 20 per cent with both the pulmonary and the brachial arterial flows. Further, in the group of abnormal subjects the agreement between the Fick and the right ventricular output was maintained. But in this latter group the flow calculated from the curve inscribed at the brachial artery exceeded the Fick value by more than 20 per cent in 5 of the 13 comparisons. These data suggest that the blood from the bronchial vessels has a higher saturation than that of the mixed venous blood.

It must be emphasized that the methods are not precise and that the anastomotic flow is, in most instances, probably small. But it appears that the possibility that

such anastomoses carry blood into the pulmonary vessels is a real one and that in some cardiopulmonary diseases the Fick more nearly measures the pulmonary arterial flow.

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*COCHLEAR POTENTIALS IN THE CAT IN RESPONSE TO HIGH-FREQUENCY SOUNDS**

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Partly because of its availability and partly because of its particular anatomy, the cat has been a favorite subject in auditory experiments for many years. Probably its hearing is better known to us than that of any other animal except man. In the present experiments the study of cochlear potentials in the cat is extended to the ultrasonic frequencies, up to 100,000 cycles. A few earlier observations have indicated that these potentials are elicited by tones well above man's upper limit of hearing,[1] but no systematic studies have been made.

As is well known, the ultrasonic frequencies present particular difficulties of a technical nature. Because of their short wave lengths, their acoustic behavior differs from that of ordinary sounds in many respects, and their production, control, and measurement require special methods. For the generation of tones in the range up to 100,000 cycles we have made use of a solid dielectric condenser receiver.[2] For the measurement of sound pressures in this range we have extended the calibration of one of our condenser microphones (Western Electric Type 640A) by the use of an electrostatic actuator.[3]

The measurement of cochlear potentials in this range also presents a problem, because the magnitude of these potentials is small in relation to the usual background of physiologic noise. To solve this problem, a high-frequency wave

analyzer was designed and constructed and used as a selective voltmeter.[4] This instrument was valuable also in the measurement of the output of the condenser microphone in the high-frequency range where this microphone presents an unfavorable signal-to-noise ratio.

The experiments were carried out mainly in young cats, though a few older ones were included for comparison. The anesthetic was diallylbarbituric acid and ethyl carbamate (Dial), in a dosage of 1 cc/kg of body weight. The recording electrode was a platinum foil on the round window membrane, with an indifferent electrode in the masseter muscle.

Some of the results are given in Figure 1, represented as the sound pressure

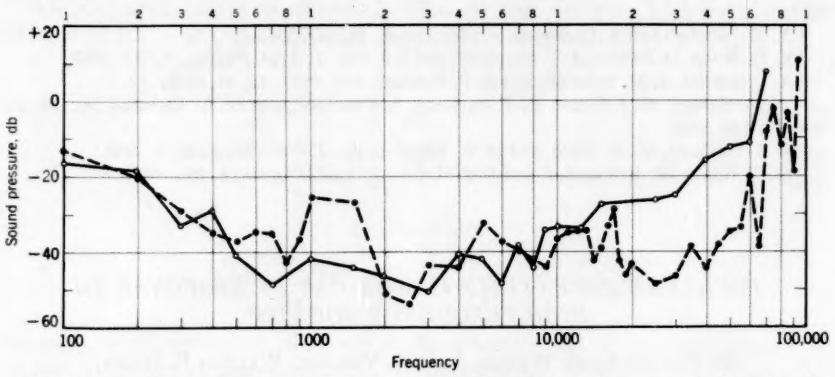

FIG. 1.—Equal-response curves for two cats, representing the sound pressure, in decibels relative to 1 dyne/sq cm, required to produce a cochlear potential of 1 microvolt.

required to produce a response of 1 microvolt. The solid curve represents the responses of a fairly old cat ($4^1/_2$ years), whereas the dashed curve represents those of a young one (about 1 year). There is little difference in sensitivity in the lower range, up to 15,000 cycles, but a significant variation beyond. The older cat showed a decline in sensitivity that became marked above 30,000 cycles and ceased to give any detectable responses above 70,000 cycles. The younger one, on the other hand, continued to show fairly uniform sensitivity up to 50,000 cycles, and only thereafter suffered a decline to a final value at 95,000 cycles, which was the highest frequency used. Most of the young animals gave curves like the dashed one shown here and usually extended to 100,000 cycles, which was the limit of the apparatus.

The maximum amount of cochlear potential at the uppermost frequencies was small, often no more than 1 microvolt and sometimes less. Yet, within the range within which measurements could be made, the intensity functions for the high frequencies show the same form as for the low frequencies: the functions are linear over their main course and then bend as overloading enters for intense sounds.

These relations may be seen in Figure 2. Here a special sort of plotting has been used to facilitate a comparison of the forms of the curves for the different frequencies. The abscissa represents sound pressure in decibels relative to 1 dyne/sq cm for a tone of 1,000 cycles, and the curves for the other frequencies have been shifted

along the abscissa until their straight portions coincide with the 1,000-cycle curve. This procedure neglects variations in sensitivity and brings out especially the difference in level at which overloading occurs.

There is a systematic decline in the maximums as the frequency is raised. Thus the observations extend those already obtained for the ordinary range of tones, which have been interpreted as revealing the forms of displacement of the basilar membrane as a function of frequency and specifically as indicating that the displacement curves grow continually narrower and their peaks shift basalward as the frequency is raised.[5] The present results are not sufficient to determine whether the displacement curves for these uppermost tones have a true maximum that shifts with frequency or whether merely the tail of the usual response curve exists at the extreme basal end and declines in magnitude as the frequency rises.

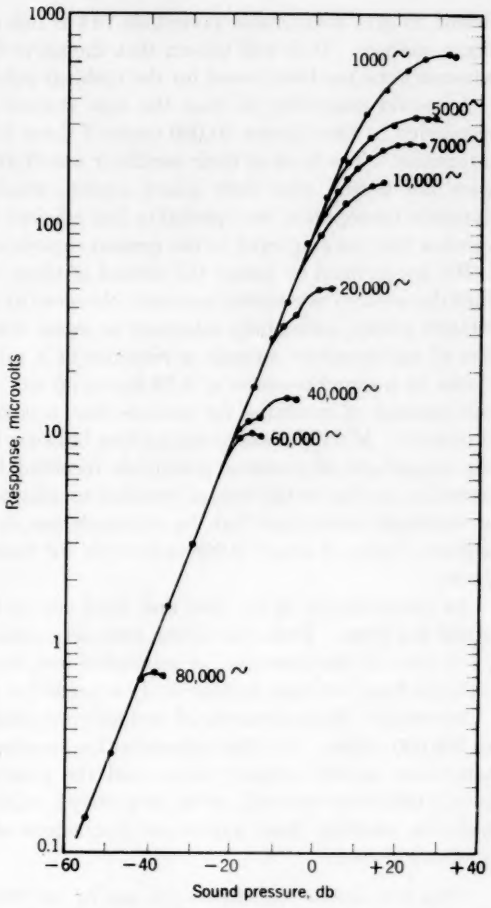

FIG. 2.—Intensity functions for a number of tones from 1,000 to 80,000 cycles. The abscissa represents sound pressure in decibels relative to 1 dyne/sq cm for the 1,000-cycle tone. The functions for all the other tones have been shifted along the abscissa so that their straight portions coincide with the 1,000-cycle function.

These results show that tones as high as 100,000 cycles enter the cat's ear and are carried by its conductive mechanism to the sensory cells of the cochlea, where they produce electrical potentials. There is still a question whether these cochlear processes are able to affect the auditory nerve fibers and bring about a perception of the sounds. Of particular relevance to this question are the results of Neff and Hind, who used a training method and measured auditory thresholds in cats for tones over a wide range.[6] They found a maximum of sensitivity around 8,000 cycles, good sensitivity as far as 50,000 cycles and an upper limit at 60,000 cycles. The correspondence with the cochlear potential curves shown here is of interest, with only a difference in the upper limit attained.

This difference can be explained in either of two ways. It may be that in the highest frequencies, those above 60,000 cycles, the level of cochlear activity is suf-

ficient to give measurable potentials but is not capable of exciting the auditory nerve endings. It is well known that the nerve fibers have excitatory thresholds, whereas none has been found for the cochlear potentials.

A second possibility is that the cats studied by Neff and Hind would have responded to tones above 60,000 cycles if these tones had been available at higher intensities. The limit of their oscillator was 70,000 cycles, and so the higher tones were not tried. Also their sound source, which was a Western Electric 633A dynamic microphone, was probably less efficient in the high frequencies than the speaker that we employed in the present experiments.

We are inclined to accept the second of these two explanations. Our reason is that the amount of cochlear potential observed at the highest frequencies, including 100,000 cycles, seems fully adequate to excite the auditory nerve fibers. Thus in one of our sensitive animals a response of 1 microvolt was obtained at 100,000 cycles at a sound pressure of 0.24 dynes/sq cm. Certainly, for lower frequencies this amount of potential far exceeds that produced by a tone at the behavioral threshold. McGill[7] made a comparison between behavioral thresholds in cats and the magnitude of cochlear potentials recorded from the round window. These potentials are below the level of practical measurement when the tones are presented at threshold intensities, but, by extrapolation, it appears that they have a fairly uniform value of about 0.006 microvolt for tones over the range of 500–10,000 cycles.

An extrapolation of the Neff and Hind curves to 100,000 cycles gives a value of about 0.5 dyne. From the above considerations we should expect that a 100,000 cycle tone of this intensity, or somewhat less, would be audible to the cat. It is obvious, however, that further study is needed to decide this question.

Summary.—Measurements of cochlear potentials in the cat have been extended to 100,000 cycles. For the uppermost frequencies the intensity functions have the same form as for ordinary tones, and the maximum values continue to decline, from 1,000 cycles onward, as the frequency is raised. Consideration is given to the question whether these uppermost frequencies affect the neural elements of the auditory system.

* This investigation was supported in part by the Office of Naval Research, under Contract Nonr 1858(18), and by Higgins funds allotted to Princeton University. Permission is granted for reproduction and use by the United States government.

[1] E. G. Wever and C. W. Bray, "A Comparative Study of the Electrical Responses of the Ear," *Proc. Am. Philos. Soc.*, **78**, 407–410, 1937.

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[7] Thomas E. McGill, *Auditory Sensitivity and the Magnitude of the Cochlear Potential* (Dissertation; Princeton University, 1958).

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